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FOREWORD

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INTRODUCTION

Retinoids, retinoic acid (RA) and its analogs, are promising chemopreventive agents against breast cancer. Their efficacy against mammary carcinogenesis in animal models has been demonstrated by their ability to increase the latency period for tumor appearance and decrease the number of animals with cancer (1). Despite their cancer preventive effects, retinoids tested in clinical trials have not yet caused major clinical responses (2, 3), implying that their anti-cancer effectiveness diminishes in more malignant cells and leads to one of the major drawbacks in retinoid therapy, the retinoid resistance. The ineffectiveness of retinoids in the treatment of patients with advanced breast cancer is consistent with in vitro observations that the anti-cancer effects of RA are mainly seen in estrogen-dependent breast cancer cells and that, upon progression of the disease to estrogen independence, breast cancer cells become refractory to RA.

The effects of retinoids are mainly mediated through the three types (α , β , and γ) of the RA receptors (RARs) and retinoid X receptors (RXRs), which function in vivo mainly as RXR/RAR heterodimers (4-6). The RXR/RAR heterodimers are activated by RAR ligands. Binding of RXR ligands can induce RXR homodimer formation and function (6) and may be required for activation of certain RXR-containing heterodimers, such as RXR/LXR (7) and RXR/nur77 (8, 9). The dimeric complexes of retinoid receptors function as transcriptional factors that bind to specific sequences on target genes and regulate the transcriptional expression of the genes. One of the most potent target genes identified so far is RAR β gene. RAR β expression is induced by RA through a RA response element (β RARE) in its promoter (10). The auto-induction of RAR β expression may play a critical role in amplifying retinoid responses.

Despite intensive research, the molecular mechanisms by which retinoids exert their anti-cancer effects and how their activities are lost in certain breast cancer cells remain unclear. The major goal of the proposed experiments is to understand the molecular mechanism by which retinoids exert their anti-cancer effects in breast cancer cells. The specific aims of this project are: 1) to analyze the anti-estrogen effect of retinoid receptors; 2) to characterize proteins that interact with RXR; 3) to analyze transactivation and anti-AP-1 activities of retinoid receptors; 4) to analyze the mechanism by which retinoid receptor activities are impaired, and 5) to analyze the function of RXR homodimers.

BODY

Expression of RAR β is critical for the anti-cancer effect of *trans*-RA. To study the mechanism by which *trans*-RA inhibits the growth of estrogen-dependent breast cancer cell lines, we examined the expression of various retinoid receptor genes in different breast cancer cell lines by Northern blot (Liu et al., 1996; Zhang et al., 1996). We observed that RAR β expression was induced by *trans*-RA only in estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines (ZR-75-1, T-47D and MCF-7) but not in estrogen-independent, *trans*-RA-resistant breast cancer cell lines (MDA-MB-231, BT-20 and MDA-MB-468). When

RAR β gene was stably transfected and expressed in *trans*--RA-resistant MDA-MB-231 cells, the growth-inhibitory effect of *trans*--RA was recovered. Conversely, when RAR β antisense cDNA was stably transfected into *trans*--RA-sensitive ZR-75-1 cells, the cells showed reduced *trans*--RA-sensitivity. These data demonstrate that RAR β plays a critical role in mediating the growth inhibitory effects of *trans*--RA in breast cancer cells.

RAR α mediates the growth inhibitory effects of *trans*--RA probably through its induction of RAR β . Expression of RAR α is also involved in mediating the growth inhibitory effect of *trans*--RA. Several studies have shown that the expression of RAR α is relatively low in estrogen-independent, *trans*--RA-resistant breast cancer cell lines (11, 12). When we examined the expression of RAR α by Northern blot, we also found that it is expressed at low levels in *trans*--RA-resistant MDA-MB-231 and MDA-MB-468 cell lines (Liu et al., 1996). When we stably expressed RAR α in MDA-MB-231 cells, we observed that growth of stable clones was strongly inhibited by *trans*--RA (Liu et al., 1996), demonstrating that the expression of RAR α could also restore *trans*--RA sensitivity in estrogen-independent cells. In addition, we analyzed the growth inhibitory effects of a number of synthetic retinoids in MCF-7 cells in collaboration with Drs. M. Dawson and J. Fontana. Our results show that growth inhibition in MCF-7 cells correlates with retinoid binding to RAR α (Dawson et al., 1995). Together, these data demonstrate that RAR α is also involved in mediating growth inhibition in breast cancer cells. However, when we investigated the expression of RAR β in MDA-MB-231 cells stably transfected with RAR α , we found that RAR β expression was enhanced in RAR α stable clones when they were treated with *trans*--RA (Liu et al., 1996). Since RAR α can activate the β RARE present in the RAR β promoter, the growth inhibitory effect of RAR α is likely due to its induction of RAR β .

Differential growth inhibitory effects of RAR-selective and RXR-selective retinoids in estrogen-dependent and -independent breast cancer cell lines. We showed that activation of RARs, but not RXRs is required for *trans*--RA-induced growth inhibition in estrogen-dependent ZR-75-1 cells (Liu et al., 1996). To further investigate the role of RARs and RXRs in breast cancer cells, we used a number of RAR-selective (SR11365, SR11383, SR11278, SR11281 and SR11277) and RXR-selective (SR11237, SR11246, SR11235, and SR11345) retinoids to determine their growth inhibitory effects in both estrogen-dependent (ZR-75-1) and estrogen-independent (MDA-MB-231) cells. RAR-selective retinoids strongly inhibited the growth of ZR-75-1 cells, but not estrogen-independent, *trans*--RA-resistant MDA-MB-231 cells (Figure 1). In contrast, RXR-selective retinoids did not affect ZR-75-1 cell growth, but significantly inhibited the growth of MDA-MB-231 cells. RXR-selective SR11246 also induced apoptosis in estrogen-independent cells but not in estrogen-dependent cells, whereas RAR-selective SR11365 but not RXR-selective SR11246 induced apoptosis only in ZR-75-1 cells (Figure 2). These data demonstrate that activation of RXR can induce growth inhibition and apoptosis in *trans*--RA-resistant breast cancer cell. Interestingly, combination of either RXR-selective retinoid with the RAR-selective retinoid SR11365 strongly enhanced the growth inhibitory effect of the RXR-selective retinoids. Thus, different retinoid signaling pathways can mediate retinoid-induced growth inhibition in estrogen-dependent and -independent breast cancer cells.

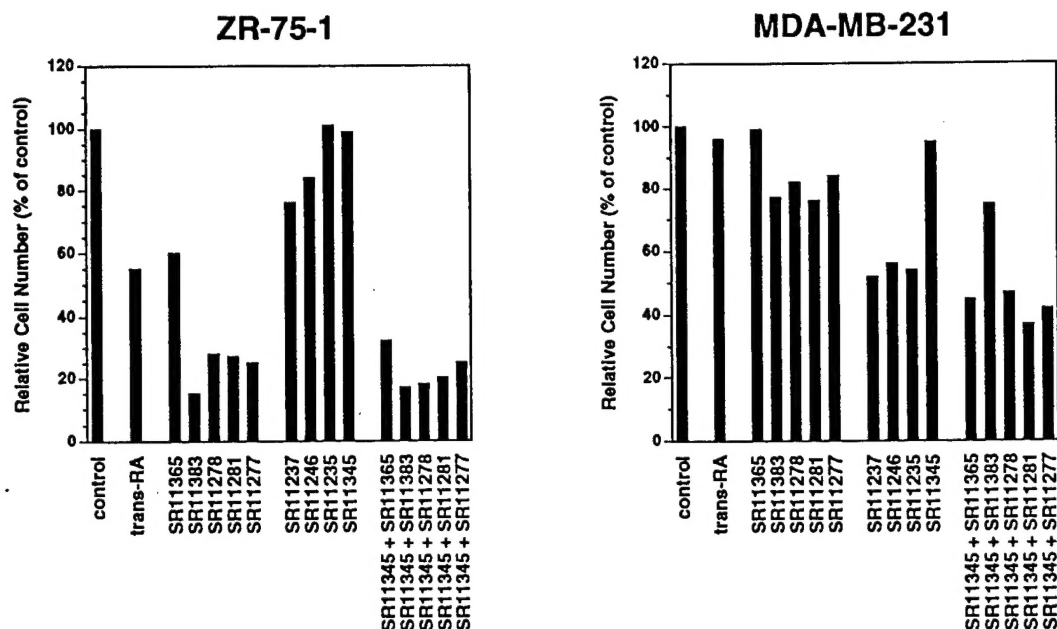


Figure 1. Growth inhibitory effect of retinoids on estrogen-dependent ZR-75-1 and estrogen-independent MDA-MB-231 breast cancer cells. Cells (800 cells/well) were seeded in 96-well plates and treated with the indicated retinoids (10^{-6} M) alone or in combination for 10 days. The number of viable cells was determined by the MTT assay.

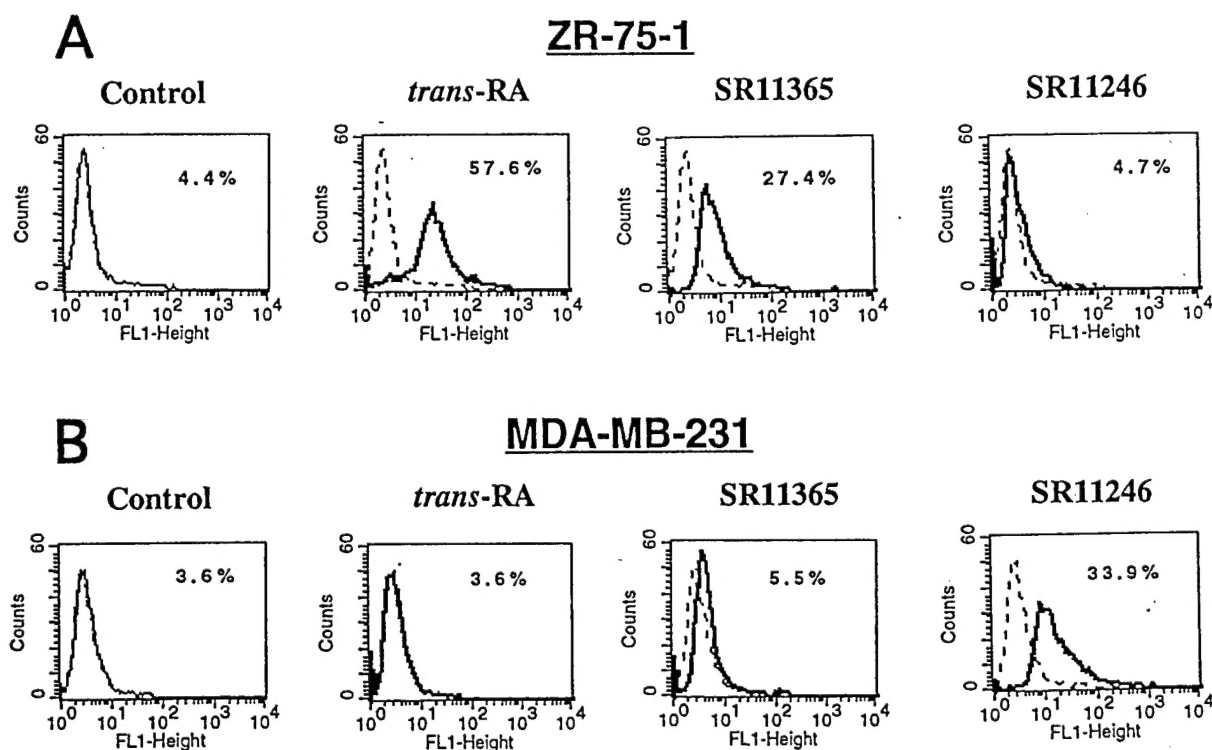


Figure 2. Induction of apoptosis by retinoids in estrogen-dependent ZR-75-1 and estrogen-independent MDA-MB-231 cells. **A.** Induction of apoptosis in ZR-75-1 cells. **B.** Induction of apoptosis in MDA-MB-231 cells. Breast cancer cells were grown in the presence of the indicated retinoids (10^{-6} M) for 48 h. DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell number. FL, fluorescence.

Expression of RAR β correlates with growth inhibitory effect of receptor-selective retinoids. To determine whether the growth inhibitory effects of receptor-selective retinoids are also mediated by expression of RAR β , we analyzed RAR β gene expression in both estrogen-dependent and -independent breast cancer cell lines in response to RAR-selective and RXR-selective retinoids (Figure 3). *Trans*-RA and RAR-selective SR11365 strongly induced RAR β expression in estrogen-dependent ZR-75-1 and T-47D cells, while RXR-selective SR11246 and SR11345 did not (Figure 3). In contrast, both RAR-selective and RXR-selective retinoids could slightly induce RAR β in estrogen-independent MDA-MB-231 cells. The combination of RAR-selective and RXR-selective retinoids induced RAR β to a level comparable to that observed in T-47D cells. Thus, our data demonstrate that RAR β induction correlates with growth inhibition and suggest that RAR β induction may contribute to growth inhibition by RXR-selective retinoids in estrogen-independent breast cancer cells.

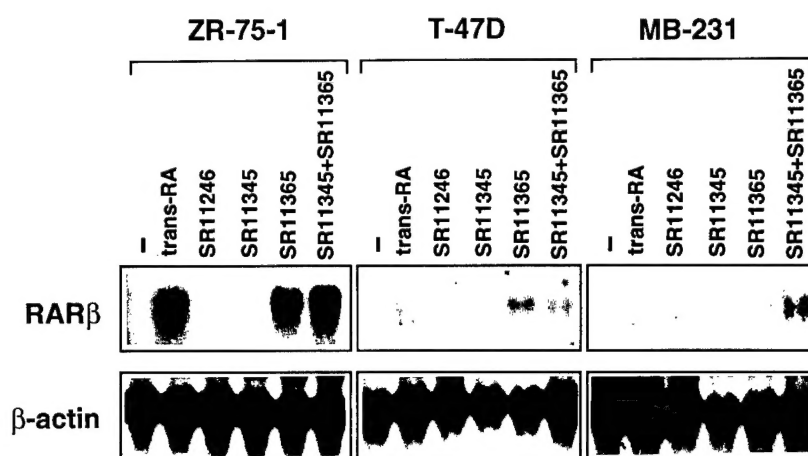


Figure 3. Effect of RAR-selective SR11365 and RXR-selective SR11246 and SR11345 on RAR β gene expression in estrogen-dependent ZR-75-1 and T-47D and estrogen-independent MDA-MB-231 cells. Cells were treated with the indicated retinoids (10^{-6} M) or in combination and analyzed for the RAR β expression by Northern blot. The expression of β -actin was used as a control.

Activation of the β RARE by RXR-selective retinoids through RXR/nur77 heterodimers. The β RARE in the RAR β gene promoter mediates RAR β expression (10). The β RARE is activated by the RXR/RAR heterodimer in response to RAR-selective retinoids, but not RXR-selective retinoids (8, 13). Recently, orphan receptor nur77 was shown to bind to DR-5 type RAREs as RXR/nur77 heterodimers (8, 9). We investigated the binding of nur77 on the β RARE. Nur77 alone did not show clear binding, but when mixed with RXR α , strong binding to β RARE occurred (Figure 4). The complex could be upshifted by anti-nur77 antibody and abolished by anti-RXR antibody, demonstrating that the complex is a RXR/nur77 heterodimer. Thus, the β RARE is an unique RARE that allows interaction with both RXR/nur77 and RXR/RAR heterodimers.

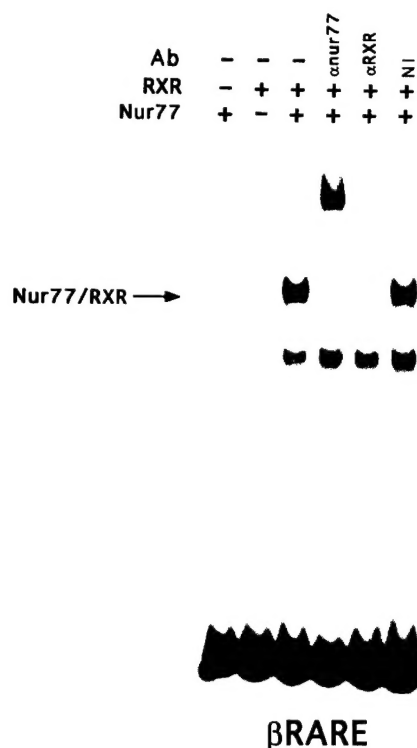


Figure 4. Nur77 forms heterodimers with RXR on the β RARE. Equal amounts of *in vitro* synthesized nur77 and RXR were incubated alone or together at room temperature for 10 min. The reaction mixtures were then incubated with 32 P-labeled β RARE and analyzed by the gel-retardation assay. When antibody (Ab) was used, it was incubated with receptor protein for 30 min at room temperature before performing the gel-retardation assay.

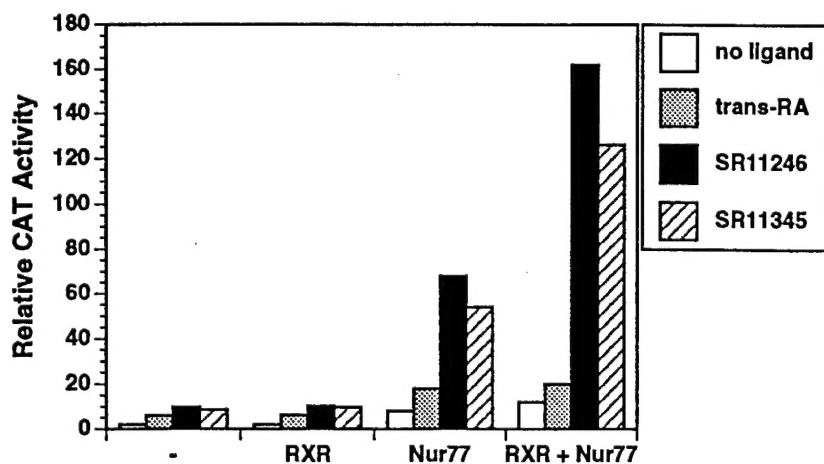


Figure 5. Nur77 promoted transactivation of the RAR β promoter by RXR-selective retinoids in CV-1 cells. A CAT reporter containing the RAR β promoter (10) was transiently transfected into CV-1 cells with the indicated receptor expression vectors. Then 24 h later, cells were treated with the indicated retinoid for 24 h. CAT reporter activities were determined as described (11).

To determine whether binding of the RXR/nur77 heterodimers to the β RARE could activate the response element in response to RXR-selective retinoids, we performed a transient transfection assay (Figure 5). Cotransfection of nur77 and RXR expression vectors significantly enhance the RAR β promoter activity in response to RXR-selective retinoids SR11246 and SR11345. This data is consistent with previous observation (8, 9) that RXR/nur77 heterodimers can be activated by RXR-selective retinoids and suggests that induction of RAR β by RXR-selective retinoids through RXR/nur77 heterodimers may be the mechanism by which RXR-selective retinoids inhibit the growth of MDA-MB-231 cells.

Levels of RAR α modulates retinoid signaling switch in breast cancer cells. Many estrogen-dependent and -independent breast cancer cell lines have similar expression levels of RAR γ and RXR α (11, 12). Nur77 expression levels are similar in ZR-75-1 and MDA-MB-231 cells [data not shown]. However, RAR α is only highly expressed in the estrogen-dependent breast cancer cell lines (11, 12). This suggests the possibility that expression of RAR α may allow preferential formation of RAR α /RXR heterodimers in estrogen-dependent breast cancer cell lines, that function to mediate the growth inhibitory effect of RAR-selective retinoids but not RXR-selective retinoids. In contrast, low expression level of RAR α in estrogen-independent breast cancer cell lines may permit formation RXR/nur77 heterodimers that can be activated by RXR-selective retinoids to induce RAR β . When we stably expressed RAR α in MDA-MB-231 cells, the growth inhibition of RAR-selective retinoids was enhanced while the effect of RXR-selective retinoids was suppressed (Figure 6). When the expression of RAR β was analyzed in the stable clones, the RAR β was strongly induced by RAR-selective retinoids but not RXR-selective retinoids (Figure 7), similar to that observed in estrogen-dependent breast cancer cell lines (Figure 3). Therefore, depending on the RAR, RXR, and nur77 levels present in cancer cells, either the *trans*-RA- or 9-*cis* RA-signaling pathway can activate the β RARE (Figure 8). This retinoid signaling switch may play an important role in regulating cell growth in response to different stimuli and suggests that low expression of RAR α may allow RXR ligand signaling but cause RAR ligand resistance.

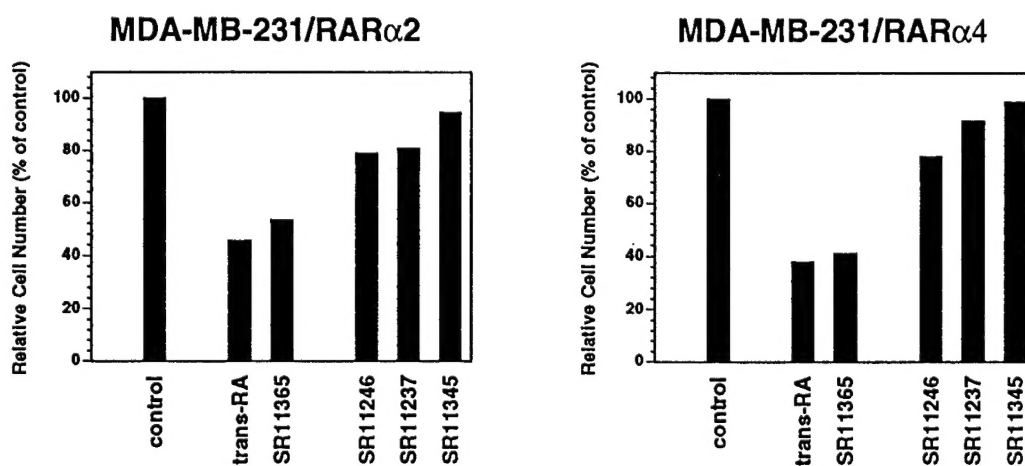


Figure 6. Effect of RAR α on sensitivity of estrogen-independent MDA-MB-231 cells to receptor-selective retinoids. Stable clones, which expressed introduced RAR α , were seeded at 800 cells/well in 96-well plates and treated with the indicated retinoid (10^{-6} M) alone or in combination for 10 days. The number of viable cells was determined by the MTT assay.

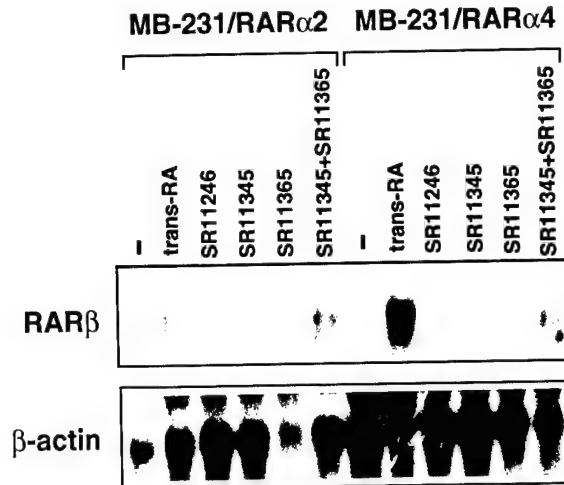


Figure 7. Effect of stable expression of RAR α on RAR β expression in estrogen-independent MDA-MB-231 cells. Stable MDA-MB-231 clones expressing high levels of RAR α (MB-231/RAR α 2 and MB-231/RAR α) were treated with the indicated retinoid (10^{-6} M) or a combination and analyzed for the expression of RAR β by Northern blot. The expression of β -actin was used as a control.

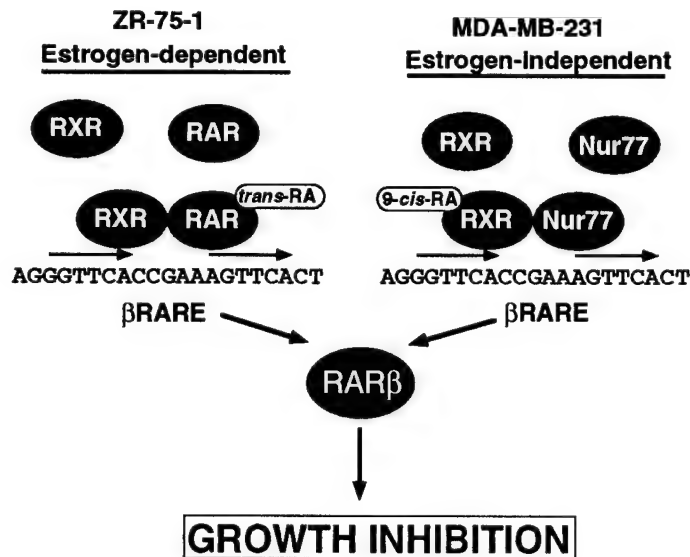


Figure 8. Retinoid signaling pathways in breast cancer cells. RAR β expression can mediate the growth inhibitory effect of retinoids and is regulated by the β RARE in RAR β promoter. β RARE bound to both RXR α /RAR α and RXR α /nur77. Relative cellular levels of RAR, RXR and nur77 may determine whether β RARE binds to RXR α /RAR α or RXR α /nur77. Estrogen-dependent breast cancer cells expressed higher levels of RAR α to favor binding of RXR α /RAR α that can be activated by RAR-selective ligands to induce RAR β expression. In contrast, estrogen-independent breast cancer cells expressed lower levels of RAR α to favor RXR α /nur77 formation, which bound to the β RARE. RXR-selective retinoids activate nur77/RXR to induce RAR β expression. Induction of RAR β by either RAR- or RXR-selective retinoids could lead to growth inhibition.

Expression of COUP-TF correlates with *trans*-RA induction of RAR β and *trans*-RA sensitivity in breast cancer cell lines. When we investigated whether expression levels of COUP-TF contribute to *trans*-RA resistance in several lung cancer cell lines, we observed that the expression of COUP-TF correlated with *trans*-RA induction of β RARE activity (Wu et al., 1996). In contrast, we found that the expression of nur77 was associated with resistance to β RARE activation by *trans*-RA. In addition, stable expression of COUP-TF in COUP-TF-negative, RA-resistant lung cancer H292 cells restored *trans*-RA sensitivity in the cells (Wu et al., 1996). Thus, the expression of COUP-TF could induce *trans*-RA responsiveness in *trans*-RA-resistant lung cancer cells. To determine whether COUP-TF could also modulate *trans*-RA sensitivity in human breast cancer cell lines, we examined the expression of COUP-TF in several breast cancer cell lines, including the *trans*-RA-resistant cell lines MB-468, BT-549, BT-20 and MB-231, as well as *trans*-RA-sensitive breast cancer cell lines T-47D and ZR-75-1. As shown in Figure 9, COUP-TF was expressed only in the *trans*-RA-sensitive breast cancer cell lines T-47D and ZR-75-1, but not in the *trans*-RA-resistant cell lines. This data, therefore, demonstrates that the expression of COUP-TF correlates with *trans*-RA induction of RAR β and *trans*-RA sensitivity and suggests that the *trans*-RA sensitivity of breast cancer cells may be also modulated by COUP-TF.

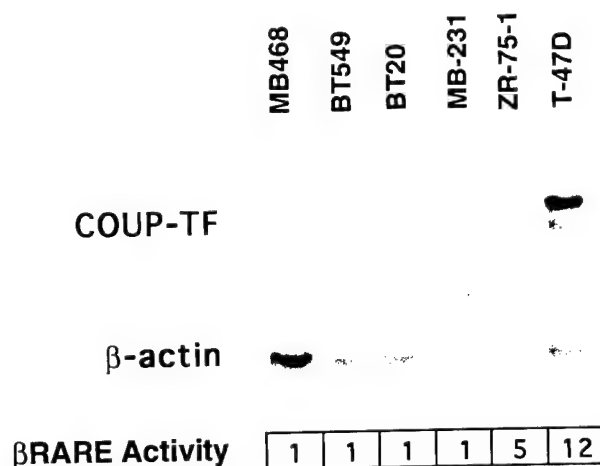


Figure 9. Expression of COUP-TF in human breast cancer cell lines. Total RNAs were prepared from the indicated human breast cancer cell lines treated with or without 10^{-7} M *trans*-RA for 24 h and analyzed for the expression of COUP-TFI by Northern blot. In the control, the expression of β -actin is shown. β RARE activity represents the fold-induction by *trans*-RA as determined by the transient transfection assay using the β RARE-tk-CAT as a reporter.

COUP-TF sensitizes β RARE *trans*-RA responsiveness. Orphan receptor COUP-TF can bind to a variety of RAREs, including β RARE (14-17). Previous studies demonstrate that COUP-TF can inhibit RA-induced activity on many RAREs by transient cotransfection assays where COUP-TF might be overexpressed (14-17). We then examined the effect of various concentrations of COUP-TF on the β RARE activity. At low concentrations (1, 5, or 10 ng), COUP-TF either did not affect or even slightly enhance the *trans*-RA-induced

β RARE activity, but did significantly inhibit the basal activity of the reporter, so that the relative increase in *trans*-RA-dependent activity was enhanced (Figure 10). These data demonstrate that COUP-TF can enhance the sensitivity of various RA target genes to *trans*-RA. We also found that nur77 could antagonize COUP-TF activity on the β RARE (Figure 10). Thus, nur77 can desensitize the responsiveness of β RARE to *trans*-RA by antagonizing COUP-TF activity.

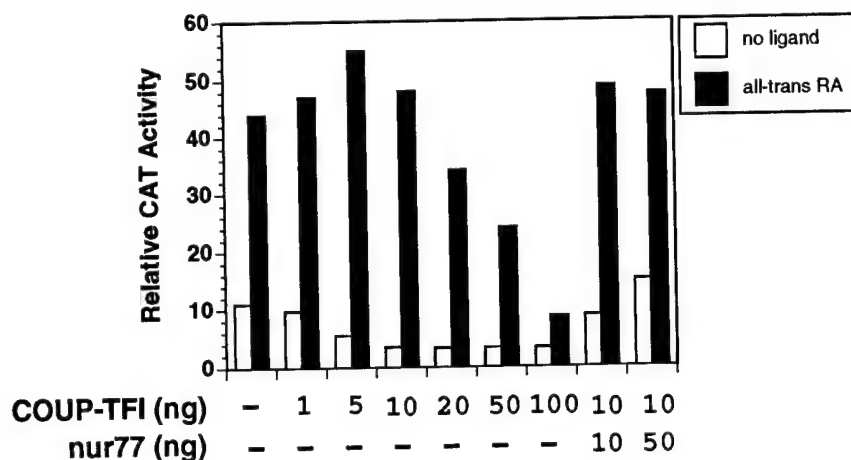


Figure 10. Modulation of *trans*-RA sensitivity of RAREs by COUP-TF and nur77 in CV-1 cells. Effect of COUP-TF and nur77 on *trans*-RA-dependent activation of β RARE. β RARE-tk-CAT was cotransfected with the indicated amounts of nur77 and/or COUP-TF into CV-1 cells. Cells were treated with or without 10^{-7} M *trans*-RA, and assayed for CAT activity.

Heterodimerization of orphan receptors COUP-TF and nur77. In the course to investigate the effect of nur77 in CV-1 cells, we observed that nur77 could inhibit COUP-TF binding on the β RARE (Figure 11). COUP-TF formed a strong complex to the β RARE (Figure 11). However, when nur77 protein was added, the COUP-TF binding complex was inhibited. The inhibition was very efficient in that a one molar excess amount of nur77 almost completely inhibited the binding, which was specific as similar amounts of RXR had no effect on COUP-TF binding. Conversely, the binding of RXR/nur77 on the β RARE was efficiently inhibited by either COUP-TFI or COUP-TFII (Figure 11). Our immunoprecipitation assay using anti-nur77 antibody and other protein-protein interaction assays (Wu et al., 1996) demonstrate that nur77 forms a stable complex with COUP-TFI or COUP-TFII in solution. These observations suggest that nur77 not only can mediate the effect of RXR-ligands through its heterodimerization with RXR (Figure 4) but also can regulate retinoid sensitivity through its interaction with COUP-TF that binds to the β RARE. We have observed that overexpression of nur77 leads to *trans*-RA resistance in lung cancer cell lines (Wu et al., 1996). However, nur77 is expressed at relative low levels in breast cancer cell lines (data not shown). It may mainly function as RXR/nur77 heterodimers to mediate the effect of RXR-ligands in estrogen-independent breast cancer cell lines since these cell lines do not express COUP-TF (Figure 9). The relationship between nur77, COUP-TF and RXR in breast cancer cell lines remains to be determined.

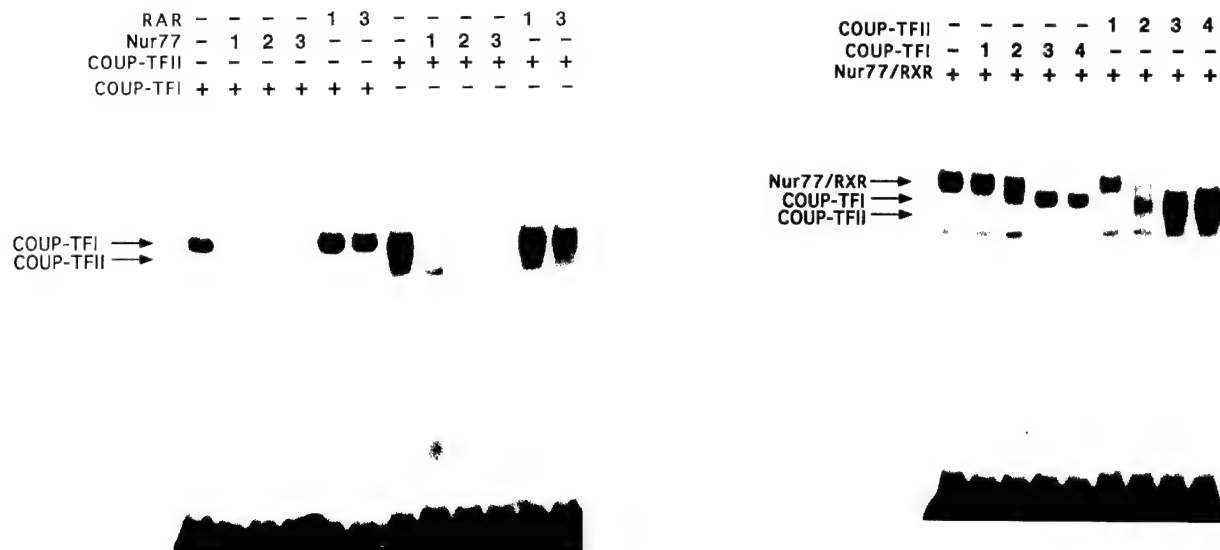


Figure 11. Mutual inhibition of nur77 and COUP-TF DNA binding. *In vitro* synthesized COUP-TF or nur77 was preincubated with the indicated molar excess of nur77 or COUP-TF, respectively. Following this preincubation, the reaction mixtures were incubated with 32 P-labeled β RARE and analyzed by the gel-retardation assay.

CONCLUSION

Research conducted in the past funding year shows several pieces of exciting findings. Our results convincingly demonstrate that expression of RAR β plays a critical role in mediating the growth inhibitory effects of *trans*-RA in both estrogen-dependent and -independent breast cancer cell lines. In addition, we show that the growth inhibitory effect of RAR α may be mediated through its induction of RAR β . The other finding in the past year is the demonstration that activation of RXR by RXR-selective retinoids can induce growth inhibition in estrogen-independent, *trans*-RA-resistant breast cancer cells. In addition, a synergistic growth inhibition effect was observed with combination of certain RAR-selective and RXR-selective retinoids. The effect of RXR-selective retinoids is probably mediated through RXR/nur77 heterodimers that bind to the β RARE and activate RAR β gene expression in response to RXR ligands. This finding is important in that we demonstrate a novel growth inhibition pathway that is mediated by activation of RXR. Importantly, the pathway is functional in RAR α -negative, estrogen-independent and *trans*-RA-resistant breast cancer cells. Thus, different retinoid growth inhibition pathways exist and different classes of retinoids can be used for different types of breast cancer. The combination treatment using a RAR-selective retinoid and a RXR-selective retinoid that synergizes could provide a means of reducing the effective dose and toxicity of retinoids. Furthermore, our studies also suggest that orphan receptor COUP-TF may play a critical role in the

modulation of retinoid sensitivity through its binding to the β RARE, which regulates RAR β expression. As proposed, during the coming year we will further investigate the molecular mechanism by which retinoid receptors function in breast cancer cells. Due to our finding of the involvement of orphan receptors nur77 and COUP-TF in regulating retinoid activity, our research will focus on the role of RXR/nur77 heterodimers in mediating RXR ligand response pathway, the involvement of COUP-TF in regulating the activity of RXR/RAR and RXR/nur77 heterodimers, and the function of RAR α expression in the switch of RAR-dependent and RXR-dependent retinoid growth inhibition response pathways.

REFERENCES

1. Moon, R. C., Mehta, R. G., and Rao, K.V.N. Retinoids and cancer in experimental animals. In *The Retinoids: Biology, Chemistry, and Medicine*, 2nd ed. M. B. Sporn, A. B. Roberts, and D. S. Goodman (Eds.), Raven Press, New York, 1994, pp. 573-595.
2. Cassidy, J., Lippman, M., LaCroix, A., *et al.* Phase II trial of 13-cis-retinoic acid in metastatic breast cancer. *Eur. J. Cancer Clin. Oncol.* **18**:925-928, 1982.
3. Modiano, M., Dalton, W., Lippman, S., *et al.* Phase II study of fenretinide (N-[4-hydroxyphenyl]retinamide) in advanced breast cancer and melanoma. *Invest. New Drugs* **8**:317-319, 1990.
4. Mangelsdorf, D. J., and Evans, R. M. The RXR heterodimers and orphan receptors. *Cell* **83**:841-850, 1995.
5. Kastner, P., Mark, M., and Chambon, P. Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell* **83**:859-869, 1995.
6. Zhang, X.-k., and Pfahl, M. Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.* **4**:156-162, 1993.
7. Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A. and Mangelsdorf, D. J. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* **9**:1033-45, 1995.
8. Forman, B. M., Umesono, K., Chen, J., and Evans, R. M. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* **81**:541-550, 1995.
9. Perlmann, T., and Jansson, L. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurr1. *Genes Dev.* **9**: 769-782, 1995.
10. Hoffmann, B., Lehmann, J. M., Zhang, X.-k., Hermann, T., Graupner, G., and Pfahl, M. A retinoic acid receptor-specific element controls the retinoic acid receptor- β promoter. *Mol. Endocrinol.* **4**:734-1743, 1990.
11. Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X.-k. RAR β mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.* **16**:1138-1149, 1996.
12. Sheikh, M. S., Shao, A.-M., Li, X.-S., Dawson, M., Jetten, A. M., Wu, S., Conley, B. A., Garcia, M., Rochefort, H., and Fontana, J. A. Retinoid-resistant estrogen receptor-negative human breast

- carcinoma cells transfected with retinoic acid receptor- α acquire sensitivity to growth inhibition by retinoids. *J. Biol. Chem.* **269**:21440-21447, 1994.
13. Kurokawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gloss, B., Rosenfeld, M. G., Heyman, R. A., and Glass, C. K. Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. *Nature* **371**:528-531.
 14. Tran, P., Zhang, X.-k., Salbert, G., Hermann, T., Lehmann, J. M., and Pfahl, M. COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol. Cell. Biol.* **12**:4666-4676, 1992.
 15. Cooney, A.J., Tsai, S.Y., O'Malley, B.W., Tsai, M.-J. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCa response elements, allowing COUP-TF to repress hormonal induction of the vitamin D₃ thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.* **12**:4153-4163, 1992.
 16. Widom, R.L., Rhee, M., Karathansis, S.K. Repression by ARP-1 sensitizes apolipoprotein AI gene responsiveness to RXR α and retinoic acid. *Mol. Cell. Biol.* **12**:3380-3389, 1992.
 17. Kliewer, S.A., Kazuhiko, U., Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A. and Evans, R.M. Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc. Natl. Acad. Sci. USA* **89**:1448-52, 1992.

APPENDIX

1. Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J., and Zhang, X.-k. RAR β mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol. Cell. Biol.* **16**:1138-1149, 1996.
2. Zhang, X.-k., Liu, Y., and Lee, M.-O. Retinoid receptors in human lung cancer and breast cancer. *Mutation Res.* **350**: 267-277, 1996
3. Dawson, M.I., Chao, W.-R., Pine, P., Jong, L., Hobbs, P.D., Rudd, C., Quick, T.C., Niles, R.M., Zhang, X.-k., Lombardo, A., Ely, K.R., Shroot, B., Fontana, J.A.. Correlation of retinoid binding affinity to RAR α with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. *Cancer Res.* **55**:4446-4451, 1995.
4. Wu, Q., Li, Y., Liu, R., Agadir, A., Lee, M.-O., Liu, Y., and Zhang, X.-k. Modulation of retinoic acid sensitivity by a dynamic balance of nur77 and COUP-TF orphan receptors and their heterodimerization. (Submitted).

Retinoic Acid Receptor β Mediates the Growth-Inhibitory Effect of Retinoic Acid by Promoting Apoptosis in Human Breast Cancer Cells

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Retinoids are known to inhibit the growth of hormone-dependent but not that of hormone-independent breast cancer cells. We investigated the involvement of retinoic acid (RA) receptors (RARs) in the differential growth-inhibitory effects of retinoids and the underlying mechanism. Our data demonstrate that induction of RAR β by RA correlates with the growth-inhibitory effect of retinoids. The hormone-independent cells acquired RA sensitivity when the RAR β expression vector was introduced and expressed in the cells. In addition, RA sensitivity of hormone-dependent cells was inhibited by a RAR β -selective antagonist and the expression of RAR β antisense RNA. Introduction of RAR α also restored RA sensitivity in hormone-independent cells, but this restoration was accomplished by the induction of endogenous RAR β expression. Furthermore, we show that induction of apoptosis contributes to the growth-inhibitory effect of RAR β . Thus, RAR β can mediate retinoid action in breast cancer cells by promoting apoptosis. Loss of RAR β , therefore, may contribute to the tumorigenicity of human mammary epithelial cells.

Retinoids, the natural and synthetic vitamin A derivatives, are known to regulate a broad range of biological processes, including growth, differentiation, and development (26, 48, 63). They are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of other human cancers (26, 47, 48, 63). The effects of retinoids are mainly mediated by two classes of nuclear receptors: the retinoic acid (RA) receptors (RARs) (4, 6, 23, 40, 62) and the retinoid X receptors (RXRs) (27, 43, 49, 50, 81). RARs and RXRs are members of the steroid-thyroid hormone receptor superfamily that also includes receptors for estrogen and vitamin D (10, 17, 25, 51, 85). Both types of retinoid receptors are coded for by three distinct genes, α , β , and γ . These receptors display distinct patterns of expression during development and differentiation (26), suggesting that each of them may have specific function. All-*trans* RA and 9-*cis* RA (28, 45), the two known active derivatives of vitamin A, essentially function as hormones by interacting with specific retinoid receptors. All-*trans* RA binds and activates RARs, and 9-*cis* RA is able to bind and activate both RARs and RXRs. RARs and RXRs modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs) (7, 37, 43, 52, 81–83, 86). Some of the target genes are RARs themselves (14, 29, 42, 44, 71), in particular the RAR β gene, for which an RARE (β RARE) that mediates RA-induced RAR β gene expression in many different cell types was identified in its promoter region (14, 29, 71). Auto-regulation of the RAR β gene presumably plays a critical role in amplifying the RA response.

Altered nuclear receptor activities are known to be associated with carcinogenesis. In human acute promyelocytic leukemia cells, an abnormal RAR α transcript is produced by chromosomal translocation (13, 36). The involvement of RAR β in cancer development was originally suggested by the finding that it was integrated by hepatitis B virus in human hepatoma (12). Recently, it was found that RAR β was not expressed in a number of malignant tumors, including lung carcinoma, squamous cell carcinoma of the head and neck, and breast carcinoma (22, 30, 32, 60, 72, 84). Given the fact that retinoids are key players in the regulatory network of cell differentiation and proliferation (26, 48, 63), altered retinoid receptors can result in abnormal cellular differentiation pathways and a loss of their antiproliferating effect, such as anti-AP-1 activity (67, 79). Recently, several studies have reported that retinoids can induce apoptosis in several different cell types (58, 61, 68). Apoptosis, a programmed cell death, is an important physiologic process in normal development and tissue homeostasis and functions as an autonomous suicide pathway that restricts cell numbers (19, 74). Induction of apoptosis by retinoids may represent an important mechanism by which retinoids inhibit cancer cell growth. Alteration of retinoid receptor activity may therefore lead to suppression of apoptosis and result in the pathological accumulation of aberrant cells and diseases involving tumors.

A considerable volume of human and animal data has suggested that retinoids are novel agents for the prevention and treatment of breast cancer (11, 33, 56). In animals, administration of retinoids inhibits the initiation and promotion of mammary tumors induced by carcinogens (11, 56). In vitro, retinoids have been shown to inhibit the growth of human breast cancer cells (20, 21, 39, 53, 65, 69, 70, 73, 75–77). Growth inhibition of such cells in culture has been observed when retinoids are administered alone or in combination with other agents, such as antiestrogen (20, 39) or interferon (78), with which synergistic effects have been observed. On the basis

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of these results, several clinical trials with retinoids have been carried out (5, 9, 55). Unfortunately, these early clinical trials with patients with advanced breast cancer have not demonstrated any significant effect, except that some benefits were observed when retinoids were used together with antiestrogen (5).

The observations that retinoids are effective in the prevention of breast cancer development and that activity is lost in patients with advanced breast cancer suggest that there is a loss of retinoid sensitivity during the progression of a breast tumor. This loss is also supported by *in vitro* observations that the growth-inhibitory effects of retinoids are mainly seen in hormone-dependent, estrogen receptor (ER)-positive breast cancer cells and that hormone-independent, ER-negative cells are refractory to the retinoid effect (20, 75). How retinoids inhibit the growth of hormone-dependent breast cancer cells and how their inhibitory effect is lost in hormone-independent cells remain largely unclear. In this study, we have determined the expression of RARs in a number of hormone-dependent and -independent breast cancer cell lines in the absence and presence of RA. Our results demonstrated that the expression of the RAR β gene was dramatically induced by RA in hormone-dependent but not in hormone-independent breast cancer cell lines. Induction of RAR β by RA correlated with the growth-inhibitory effect of retinoids in the cell lines investigated. The requirement of RAR β expression for the RA-induced growth inhibition was further demonstrated by restored RA sensitivities in hormone-independent cells after the introduction of RAR β and by diminished RA sensitivities in hormone-dependent cells because of an RAR β -selective antagonist and the expression of RAR β antisense RNA. In addition, our data demonstrated that RAR β could promote apoptosis in breast cancer cells. Thus, the loss of RAR β gene expression could be one of the major factors responsible for the loss of RA sensitivities in breast cancer cells and may contribute to their transformed phenotype.

MATERIALS AND METHODS

Cell culture. Breast cancer cell lines ZR-75-1, T-47D, MB231, BT-20, and MB468 were obtained from the American Type Culture Collection. MCF-7 was obtained from S. Sukumar (Salk Institute, La Jolla, Calif.). ZR-75-1 and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), MB231, MCF-7, and MB468 cells were grown in Dulbecco modified Eagle medium supplemented with 10% FCS, and BT-20 cells were maintained in minimal essential medium with 10% FCS.

Growth inhibition assay. To study anchorage-dependent growth, cells were seeded at 1,000 to 2,000 cells per well in 96-well plates and treated with various concentrations of retinoids. Media were changed every 48 h. The number of viable cells was determined by measuring their capacity to convert a tetrazolium salt into a blue formazan product with a nonradioactive cell proliferation and cytotoxicity assay kit (Promega, Madison, Wis.) (57). For the anchorage-independent growth assay, 30,000 cells in culture medium containing 10% FCS, 0.3% agar (Difco, Detroit, Mich.), and 10^{-7} M all-*trans* RA in a 60-mm-diameter dish were plated onto an already hardened 0.6% agar underlayer in medium supplemented with 10% FCS. The plates were incubated for 21 days with 5% CO $_2$. A colony was defined as >40 cells, and colonies with more than 40 cells were counted with a microscope.

RNA preparation and Northern (RNA) blot. For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method (66). About 30 μ g of total RNAs from different cell lines was fractionated on a 1% agarose gel, transferred to nylon filters, and probed with the 32 P-labeled ligand-binding domain of RAR cDNAs as previously described (84). To determine that equal amounts of RNA were used, the filters were also probed with rRNA L32 cDNA.

Preparation of nuclear extracts and gel retardation assays. Nuclear extracts were prepared essentially according to the method previously described (41). Briefly, cells growing to about 90% confluence were washed with cold phosphate-buffered saline (PBS) and scraped into PBS with a rubber policeman. Cells were pelleted by low-speed centrifugation and then resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM CaCl $_2$, and 2 mM MgCl $_2$. After they were pelleted, the cells were lysed in the buffer containing 1% Nonidet P-40 by 10 to 15 strokes with an ice-cold Dounce homogenizer. Immediately after lysis, nuclei were collected by centrifugation at $2,000 \times g$ and washed once with a buffer

containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.9), 1.5 mM MgCl $_2$, 10 mM KCl, and 0.5 mM dithiothreitol. Nuclear proteins were extracted with a high-salt buffer containing 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl $_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol. All the buffers used for the procedure contained protease inhibitors, i.e., 100 μ g of phenylmethylsulfonyl fluoride per ml, 1 μ g of leupeptin per ml, and 1 μ g of aprotinin per ml. When it was necessary, nuclear extracts were concentrated with a Centricon 10 microconcentrator (Millipore). Small aliquots of nuclear proteins were immediately frozen and kept at -80°C until use. To study β RARE binding, nuclear extracts were prepared from different breast cancer cell lines treated or not treated with 10^{-6} M all-*trans* RA. Nuclear extracts (5 μ g) from different breast cancer cells were analyzed by gel retardation assays for their β RARE binding activity, with 32 P-labeled β RARE being used as a probe as described previously (41). β RARE used in the experiments is the direct repeat of the RARE present in the RAR β promoter (AGGGTTCAGGCAAAGTTCAC). Labeled DNA probes were purified by gel electrophoresis and used for the gel retardation assay.

Transient transfection and CAT assay. To measure the transcriptional activation of β RARE in breast cancer cell lines, β RARE linked with the chloramphenicol acetyltransferase (CAT) gene (β RARE-tk-CAT) was used as a reporter gene to determine the RA response in hormone-dependent and -independent human breast cancer cell lines. β RARE-tk-CAT (2.0 μ g) and 3.0 μ g of β -galactosidase expression vector (pCH 110; Pharmacia) were transiently transfected into cells by the calcium phosphate precipitation method (41). Cells were grown in the presence or absence of 10^{-7} M all-*trans* RA. Transfection efficiency was normalized by β -galactosidase activity. The gathered data were shown as the means of three separate experiments.

Stable transfection. To construct RAR β and RAR α expression vectors, cDNA for the RAR β or RAR α gene was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, Calif.). To construct the RAR β antisense expression vector, cDNA for the RAR β gene was cloned into the pRc/CMV expression vector in an antisense orientation. The resulting recombinant constructs were then stably transfected into breast cancer cells by the calcium phosphate precipitation method and screened with 400 μ g of G418 (Gibco BRL, Grand Island, N.Y.). The integration and expression of exogenous RAR β and RAR α cDNA were determined by Southern blotting and Northern blotting, respectively.

Apoptosis analysis. For morphological analysis (34), cells treated with 10^{-6} M all-*trans* RA and untreated cells were trypsinized and washed with PBS. After fixation with 3.7% paraformaldehyde followed by acid-alcohol treatment, the cells were stained with propidium iodide (50 μ g/ml) containing 100 μ g of DNase-free RNase A per ml to visualize the nuclei. Stained cells were examined with a Zeiss LSM 410 confocal laser-scanning microscope. Overlays of cells were made with confocal sections at increments of 1 μ m. Apoptotic nuclei were condensed and were more brightly stained than nonapoptotic ones. For terminal deoxynucleotidyl transferase (TdT) assays (24), the cells were treated or not treated with 10^{-6} M all-*trans* RA. After 24 h, the cells were trypsinized, washed with PBS, and fixed in 1% formaldehyde in PBS (pH 7.4). After being washed with PBS, the cells were resuspended in 70% ice-cold ethanol and immediately stored overnight at -20°C . The cells were then labeled with biotin-16-dUTP by the terminal transferase method and stained with avidin-fluorescein isothiocyanate (Boehringer, Mannheim, Germany). Fluorescence-labeled cells were analyzed with a FACStar Plus. Representative histograms were made. For enzyme-linked immunosorbent assays (ELISAs), cells from breast cancer cell lines MB231, MB231/RAR β , ZR-75-1, and ZR-75-1/A-RAR β 10 were split at the same time and treated with 10^{-6} M all-*trans* RA for 12, 24, and 48 h. When antagonists (5×10^{-7} M) were used, they were incubated or not incubated with 10^{-7} M all-*trans* RA for 24 h. Treated cells were harvested at the same time. DNA fragmentation was measured with the cell death detection ELISA kit (Boehringer). About 2×10^4 cells were assayed for DNA fragmentation by following the manufacturer's protocol. The results were expressed relative to those for controls that did not receive RA treatment.

RESULTS

Activation of RARs and not RXRs is responsible for RA-induced growth inhibition in breast cancer cells. Retinoids are known to inhibit the growth of breast cancer cells. However, how the growth-inhibitory effect of retinoids is mediated is largely unknown. To establish the involvement of RAR and RXR in RA-induced growth inhibition in hormone-dependent breast cancer cells, we used retinoids selective for RXR homodimers and RXR-RAR heterodimers. Ch55 (35) and (all-E)-UAB8 (1), which specifically bind RARs and activate RXR-RAR heterodimers, displayed degrees of growth inhibition similar to that observed with all-*trans* RA in ZR-75-1 and T-47D cells, while (9Z)-UAB8 (1), which specifically activates RXR homodimers, did not show clear growth-inhibitory effects (Fig. 1). These data suggest that activation of RARs is mainly responsible for

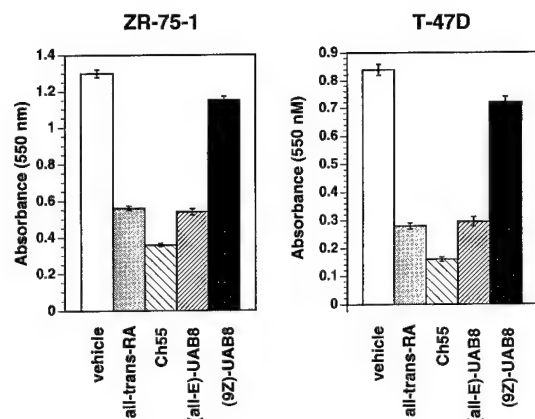


FIG. 1. Activation of RARs but not RXRs is required for RA-induced growth inhibition in breast cancer cell lines. The effects of RAR-RXR heterodimer [Ch55 and (all-E)-UAB8] and RXR homodimer [(9Z)-UAB8] specific activators on the growth of hormone-dependent breast cancer cells (ZR-75-1 and T-47D) are shown in graphic form. The effect of all-*trans* RA is shown for purposes of comparison. Cells were seeded at 1,000 cells per well and treated with 10^{-7} M retinoids for 10 days. The results were expressed as the A_{550} of MTT-derived formazan developed by cells treated with control solvent. All data shown are representative of three independent experiments. Error bars indicate standard deviations.

the RA-induced growth inhibition of ZR-75-1 and T-47D breast cancer cells, consistent with a previous observation (69).

Induction of RAR β by RA correlates with the growth-inhibitory effect of RA. To determine which RAR subtype is involved in RA-induced growth inhibition, we investigated the expression of three types of RARs (α , β , and γ) in a number of human breast cancer cell lines, including hormone-dependent (T-47D, ZR-75-1, and MCF-7) and hormone-independent (MB-468, BT-20, and MB231) lines. In a result similar to those of previous observations (64, 75), transcript for RAR γ was detected in all of the cell lines at similar expression levels (Fig. 2a). RAR α transcripts were also present in all of the cell lines. However, relatively low levels of RAR expression were found in two hormone-independent cell lines (MB231 and MB-468) (Fig. 2b). All of these cell lines did not exhibit detectable levels of RAR β mRNA under the conditions used (Fig. 2c). Since the expression of RARs could be regulated by RA because of the presence of RAREs in their promoter regions (14, 29, 42, 44, 71), we analyzed the expression of RARs in the presence of RA in these cell lines. Treatment of these cells with 10^{-6} M all-*trans* RA for 36 h did not show any effect on the expression levels of RAR α and RAR γ (Fig. 2a and b). However, the expression of RAR β was strongly enhanced by all-*trans* RA in the hormone-dependent cell lines (Fig. 2c). Surprisingly, all-*trans* RA failed to induce RAR β in hormone-independent cell lines. When the growth-inhibitory effects of all-*trans* RA and 9-*cis* RA were examined, both RAs showed strong growth inhibition with hormone-dependent cell lines, while they had little effect on hormone-independent lines (Fig. 2d). Thus, the induction of RAR β gene expression by all-*trans* RA correlates with all-*trans* RA-induced growth inhibition, suggesting that RAR β may mediate the differential growth-inhibitory effects of RA in breast cancer cells.

Abnormal transcriptional regulation of β RARE in hormone-independent breast cancer cells. All-*trans* RA-induced RAR β expression is mediated by the β RARE present in the RAR β promoter (14, 29, 71). The loss of the all-*trans* RA effect in inducing RAR β gene expression in hormone-independent human breast cancer cell lines indicates that the regulation of RAR β expression by RA is disturbed in these cells. To further

examine the impaired RA response, a CAT reporter construct containing β RARE linked with a thymidine kinase promoter (β RARE-tk-CAT) (29) was used as a reporter to determine the degrees of RA response in both hormone-dependent and -independent cancer cell lines by transient transfection assays. When this reporter was transfected into hormone-dependent cells (T-47D and ZR-75-1), a strong induction of CAT activity in response to all-*trans* RA was observed (Fig. 3a). In contrast, only a slight induction of CAT gene expression was seen in hormone-independent cells (MB231 and MB468). These results are consistent with those of a previous observation (75) and suggest that the loss of RAR β expression in hormone-independent breast cancer cells may be due to an abnormal transcriptional regulation of β RARE. To investigate whether the loss of the β RARE activity is due to altered β RARE binding, nuclear proteins from hormone-dependent and -independent breast cancer cells that were treated with all-*trans* RA or untreated were prepared and analyzed by gel retardation for their binding to β RARE. As can be seen in Fig. 3b, strong DNA binding complexes were formed when nuclear proteins prepared from ZR-75-1 or T-47D cells were used. The binding of the complexes was much stronger when nuclear proteins were prepared from cells treated with all-*trans* RA. However, complexes were hardly seen when nuclear proteins prepared from hormone-independent cells (MB468 and MB231) that were treated with all-*trans* RA or untreated were used. Thus, altered β RARE binding activity may be responsible for the loss of all-*trans* RA-induced β RARE transcriptional activation in the hormone-independent cells.

Recovery of RA sensitivity in hormone-independent breast cancer cells by RAR β expression. The data outlined above suggest that induction of RAR β by all-*trans* RA may be responsible for the RA-induced growth inhibition in hormone-dependent breast cancer cells and that the loss of RA sensitivity in hormone-independent breast cancer cells may be due to a lack of or low levels of RAR β in these cells. To directly test this, cDNA for the RAR β gene was cloned into the pRc/CMV vector so that the expression of the RAR β gene is under the control of the cytomegalovirus promoter. The vector also contains a neomycin resistance gene that allows transfected cells to grow in the presence of G418. pRc/CMV-RAR β was transfected into hormone-independent breast cancer cells (MB231). Six neomycin resistance MB231 clones that carried the exogenous RAR β gene, as revealed by Southern blot analysis (data not shown), were selected. Among these clones, MB231/RAR β 2 and MB231/RAR β 3 expressed exogenous RAR β gene transcripts (Fig. 4a), as judged by their sizes, which were smaller than that of the endogenous RAR β transcript observed in RA-treated ZR-75-1 cells (data not shown). The rest of the clones and cells transfected with pRc/CMV empty vector did not show any RAR β transcript.

To determine the effect of the introduced RAR β , the growth of MB231/RAR β 2, MB231/RAR β 3, MB231/RAR β 9, and cells transfected with empty vector was measured in the presence or absence of either all-*trans* RA or 9-*cis* RA by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 4b). For cells in the presence of RA, we observed a strong growth inhibition of RAR β -transfected cells. Treatment with 10^{-6} M RA resulted in an about 50% inhibition of MB231/RAR β 2 cell growth. Under the same conditions, about 40% inhibition was seen in MB231/RAR β 3 cells. In contrast, MB231 cells transfected with empty vector or parental MB231 cells did not show any response to RA. The fact that cells (MB231/RAR β 9) transfected with pRc/CMV-RAR β vector but failing to express RAR β (Fig. 4a) did not show any response to RA (Fig. 4b) suggests that the growth-inhibitory

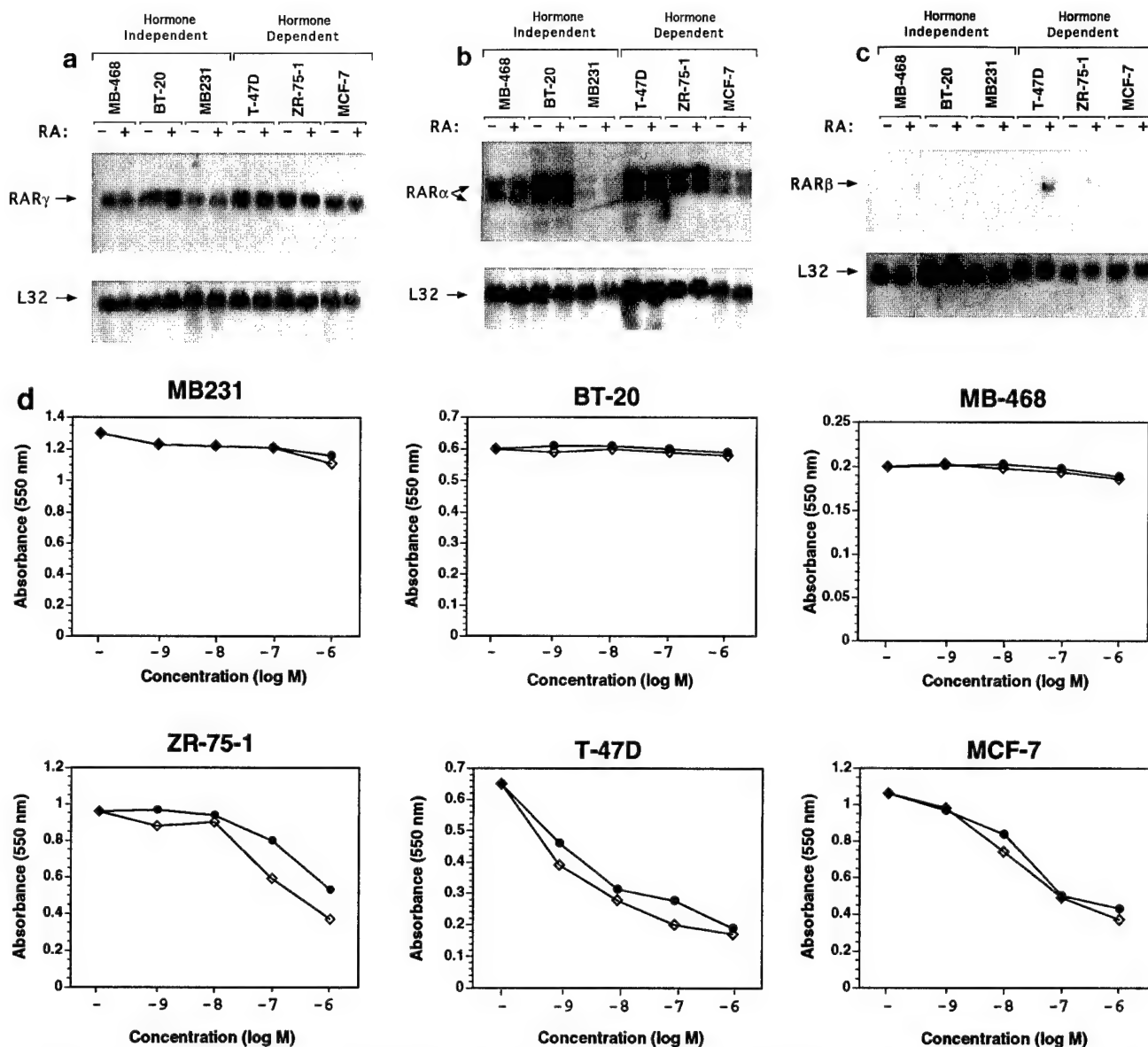


FIG. 2. Expression of retinoid receptors and growth-inhibitory effect of RA in human breast cancer cells. Expression of RAR α (a), α (b), and β (c) in hormone-dependent and -independent human breast cancer cell lines is shown. The expression of RAR genes was determined by Northern blot analysis, with total RNA (about 30 μ g) prepared from different breast cancer cell lines being used. To determine the effect of RA, cells were treated with 10^{-6} M all-*trans* RA for 36 h before RNA preparation. (d) Effect of all-*trans* RA (solid circles) and 9-*cis* RA (open diamonds) on the growth of hormone-dependent and -independent breast cancer cells. A total of 2,000 cells per well were seeded and treated with various concentrations of RA for 7 days. Growth inhibition was performed as described in the legend to Fig. 1.

effect of RA is mediated by the RAR β product. To further characterize the effect of the transfected RAR β gene, RAR β -transfected cells were analyzed for anchorage-independent growth in soft agar. As can be seen from Fig. 5, the growth of transfectant cells that expressed RAR β (MB231/RAR β 2 and MB231/RAR β 3) in soft agar was dramatically inhibited by all-*trans* RA, whereas the growth of parental MB231 cells was not affected. Together, these data demonstrate that the expression of RAR β can restore RA sensitivity in hormone-independent breast cancer cells.

Induction of RAR β by RAR α is responsible for the recovery of RA sensitivity in hormone-independent breast cancer cells. The expression of RAR α is relatively low in some of the hormone-independent cell lines, such as MB231 and MB-468 (Fig.

2b). This low expression has been previously suggested to account for RA resistance in hormone-independent cells (69, 70). We then investigated whether the expression of RAR α could restore RA sensitivity in hormone-independent cells. RAR α cDNA was cloned into the pRc/CMV vector, and the resulting expression plasmid was stably transfected into MB231 cells. Two clones (MB231/RAR α 1 and MB231/RAR α 2) that expressed introduced RAR α gene (data not shown) showed a strong RA growth-inhibitory effect in a concentration-dependent manner, while RAR α had little effect on the growth of parental MB231 cells (Fig. 6a). Since all-*trans* RA is known to activate β RARE (14, 29, 71), we therefore investigated whether expression of introduced RAR α could result in the induction of endogenous RAR β . As can be seen in Fig. 6b, the

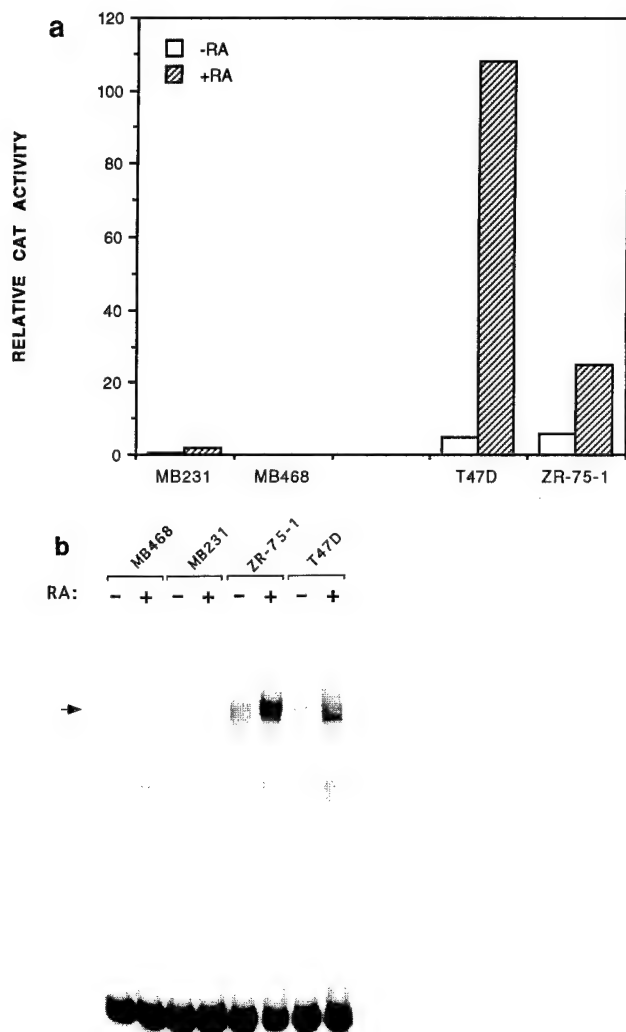


FIG. 3. Transcriptional activity and binding of β RARE in human breast cancer cell lines. (a) Transcriptional activation of β RARE in hormone-dependent and -independent human breast cancer cell lines. Transient transfection assays were used to determine transcriptional activation of β RARE in various human breast cancer cell lines. (b) β RARE binding of nuclear proteins prepared from hormone-dependent and -independent human breast cancer cell lines. The arrow indicates the specific binding complex present in breast cancer cells.

expression of endogenous RAR β was significantly enhanced in several different clones that expressed exogenous RAR α when these cells were treated with 10^{-6} M all-*trans* RA. Thus, activation of RAR α can result in the induction of RAR β in breast cancer cells. Furthermore, Ro 41-5253 (2), an RAR α -selective antagonist, strongly inhibited the induction of RAR β by all-*trans* RA in T-47D and ZR-75-1 cells (Fig. 6c). Ro 41-5253 but not LE135, an RAR β -selective antagonist (18, 46), was also able to inhibit all-*trans* RA-induced RAR β expression in MB231/RAR α 2 (Fig. 6c). Together, the growth-inhibitory effect of RAR α is likely due to its induction of the endogenous RAR β through activation of β RARE.

RAR β is essential for RA-induced growth inhibition in hormone-dependent cells. To investigate whether the induction of RAR β by RA can mediate the RA-induced growth inhibition in hormone-dependent cells, we used an RAR β -selective antagonist (LE135) (18, 46) to suppress RAR β activity in the cells. This retinoid can specifically inhibit RAR β - but not

RAR α - or RAR γ -mediated activation of target genes (46). As can be seen from Fig. 7a, a concentration-dependent reduction in RA-induced growth inhibition was observed in both ZR-75-1 and T-47D cells when LE135 was added to cells together with 10^{-7} M all-*trans* RA. In the presence of 10^{-6} M LE135, all-*trans* RA-induced growth inhibition in ZR-75-1 cells was reduced from about 50 to 20%. A similar degree of effect was seen with T-47D cells. These data suggest that activation of RAR β is mainly responsible for RA-induced growth inhibition in hormone-dependent breast cancer cells. To further support this conclusion, we stably transfected an RAR β antisense cDNA cloned into pRc/CMV into ZR-75-1 cells. Expression of antisense mRNA is known to reduce the level of its target mRNA by hybridization, which results in the degradation of the double-stranded RNA (8). As can be seen from Fig. 7b, clone A-RAR β 5 did not show detectable expression of RAR β antisense RNA. The expression of endogenous RAR β in this clone is highly induced by RA in a manner similar to that for parental cells as determined by Northern blot analysis (Fig. 7b and data not shown). In contrast, clone A-RAR β 10, which expressed RAR β antisense RNA, failed to express endogenous RAR β under all-*trans* RA treatment (Fig. 7b). When the growth-inhibitory effect of all-*trans* RA was examined in two clones that expressed RAR β antisense RNA (A-RAR β 10 and A-RAR β 25) (Fig. 7b and data not shown), we observed reduced all-*trans* RA-induced growth inhibition in these clones (Fig. 7c). In contrast, the growth of A-RAR β 5 remained strongly inhibitable by all-*trans* RA (Fig. 7c). These results, therefore, demonstrate that expression of RAR β is essential for RA-induced growth inhibition in hormone-dependent breast cancer cells.

RA-activated RAR β promotes apoptosis in breast cancer cells. When RAR β was expressed in MB231 cells, we noticed a morphological change in the cells. MB231 cells, when seeded at low density, were elongated (Fig. 8a). However, MB231 cells that expressed RAR β were relatively round and became shrunken. Such a morphological change was observed even in the absence of RA, probably because of the constitutive high level of RAR β expression and the presence of residual amounts of retinoids in the serum. The ability of the cells that expressed RAR β to survive on culture dishes was quite different from that of wild-type cells. After the continuation of culture at low density, a large portion of cells eventually died, particularly in the presence of all-*trans* RA, as assayed by the trypan blue dye exclusion method (data not shown). To investigate whether the loss of survivability of the cells is due to apoptosis, we examined the nuclear morphology of MB231 cells that expressed RAR β . When the nuclei of these cells were stained with propidium iodide and examined by confocal fluorescence microscopy, we found that many of the RA-treated MB231/RAR β 3 cells were smaller and contained condensed and fragmented nuclei with brightly stained chromatin, i.e., morphological changes typical of apoptosis (74) (Fig. 8b). RA caused similar nucleus morphological alterations in the all-*trans* RA-sensitive lines ZR-75-1, MCF-7, and T-47D (Fig. 8c, d, and e) but not in the all-*trans* RA-resistant lines MB231, MB-468, and BT-20 (data not shown).

To further study RA-induced apoptosis in breast cancer cells, we carried out TdT assays with flow cytometric analysis to study DNA fragmentation. As can be seen in Fig. 8f, the stable expression of RAR β in MB231 cells (MB231/RAR β 3) resulted in significant amounts of TdT-labeled cells when they were treated with 10^{-6} M all-*trans* RA for 24 h. Treatment of MB231 cells with all-*trans* RA did not show a clear increase of TdT-labeled cells. In ZR-75-1 cells, a marked increase in TdT-labeled cells was observed in response to all-*trans* RA. However, the TdT labeling was significantly inhibited when RAR β

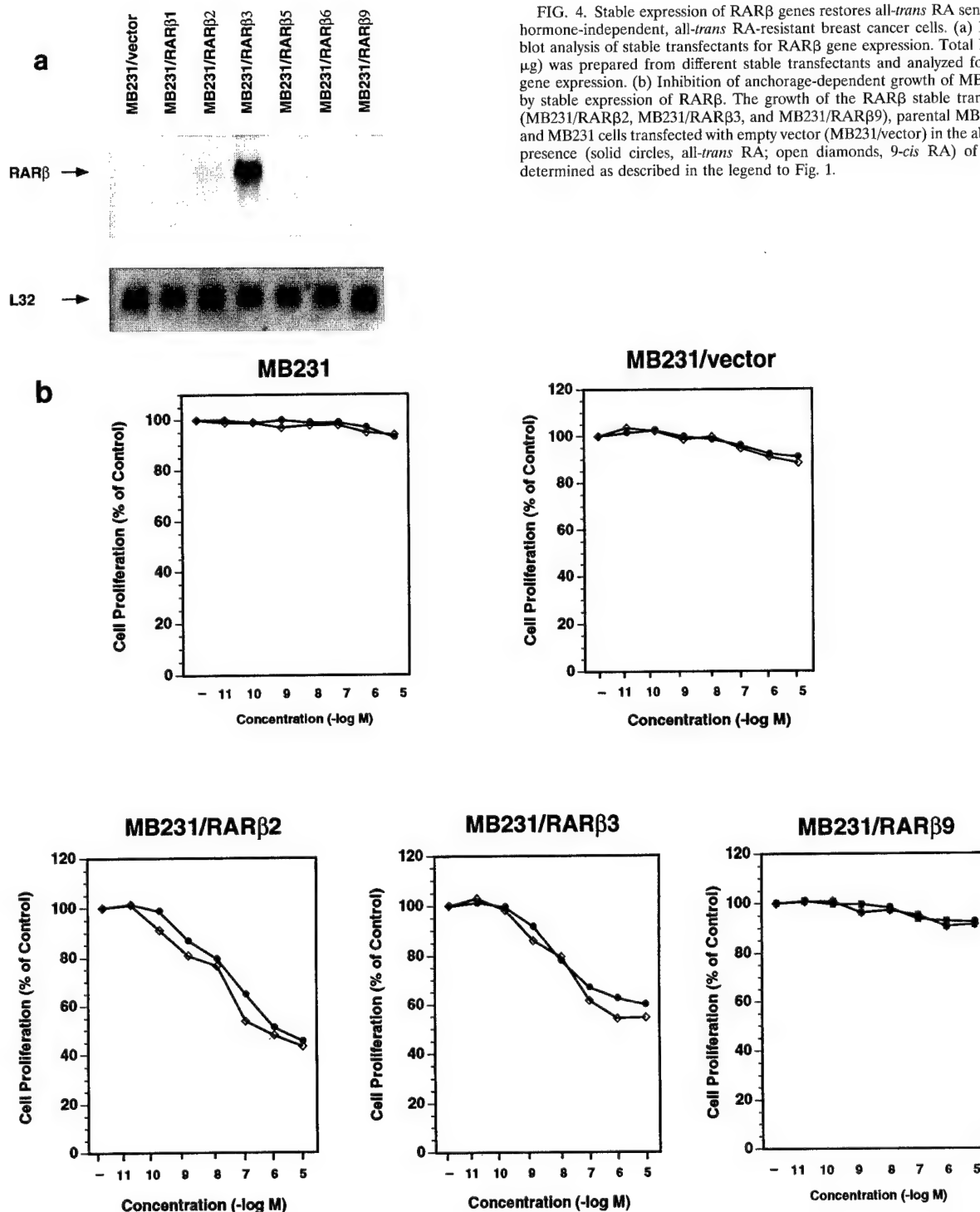


FIG. 4. Stable expression of RAR β genes restores all-*trans* RA sensitivity in hormone-independent, all-*trans* RA-resistant breast cancer cells. (a) Northern blot analysis of stable transfectants for RAR β gene expression. Total RNA (30 μ g) was prepared from different stable transfectants and analyzed for RAR β gene expression. (b) Inhibition of anchorage-dependent growth of MB231 cells by stable expression of RAR β . The growth of the RAR β stable transfectants (MB231/RAR β 2, MB231/RAR β 3, and MB231/RAR β 9), parental MB231 cells, and MB231 cells transfected with empty vector (MB231/vector) in the absence or presence (solid circles, all-*trans* RA; open diamonds, 9-*cis* RA) of RA was determined as described in the legend to Fig. 1.

antisense RNA was expressed (A-RAR β 10). Similar results were obtained by another assay (Fig. 8g), which is based on the sandwich enzyme immunoassay principle to determine cytoplasmic histone-associated DNA fragments in apoptotic cells. This study also revealed that DNA fragmentation in ZR-75-1 and MB231/RAR β 3 occurred as early as 12 h after the cells were exposed to all-*trans* RA. Furthermore, LE135 but not Ro 41-5253 was able to inhibit all-*trans* RA-induced apoptosis in

MB231/RAR β 3. Together, these results clearly demonstrate that all-*trans* RA can induce apoptosis in breast cancer cells and that all-*trans* RA-induced apoptosis is mediated by RAR β in the cells.

DISCUSSION

Retinoids are effective growth inhibitors of breast cancer cells. However, inhibition of growth is often observed in hor-

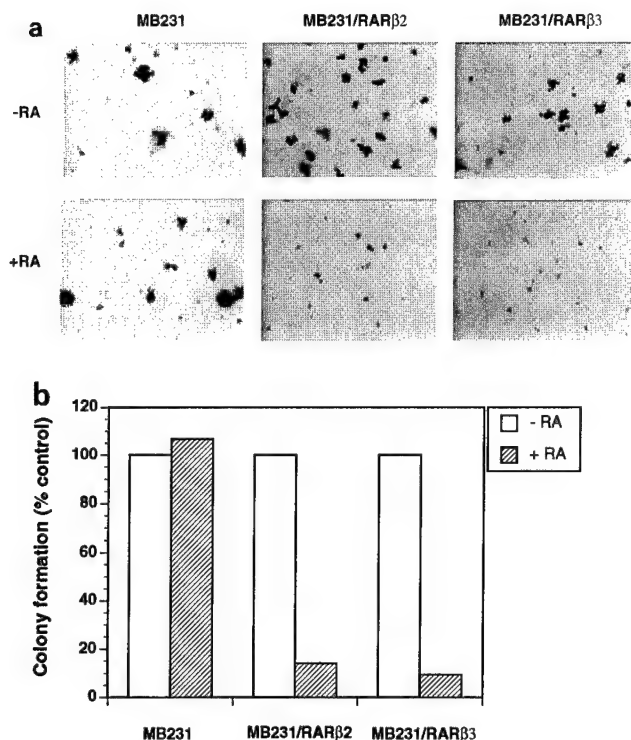


FIG. 5. Inhibition of anchorage-independent growth of MB231 cells by RAR β gene expression. (a) Photograph of colonies formed by parental MB231, MB231/RAR β 2, and MB231/RAR β 3 cells. (b) Quantitation of colonies formed by parental MB231, MB231/RAR β 2, and MB231/RAR β 3 cells. Colonies formed by MB231/RAR β 2, MB231/RAR β 3, and parental MB231 cells in the presence or absence of all-*trans* RA were scored and expressed as percentages of the number of colonies formed by cells treated with control solvent.

mone-dependent but not in hormone-independent breast cancer cells (20, 75). In this study, we have demonstrated that the expression of the RAR β gene is critical for RA-induced growth inhibition. By using receptor-selective retinoids, we first show that activation of RXRs is not involved in the cell lines (ZR-75-1 and T-47D) studied (Fig. 1). Retinoids that activate RXR and induce RXR homodimer formation did not show a clear effect on the growth of two different hormone-dependent breast cancer cell lines (ZR-75-1 and T-47D), while retinoids that activate RARs were as effective as all-*trans* RA. These data are consistent with results obtained from a previous study that used different RXR-selective retinoids in MCF-7 and other breast cancer cell lines (69). Thus, selective activation of the RXR homodimer pathway may not contribute substantially to RA-induced growth inhibition in the breast cancer cells analyzed, and activation of the RAR pathway may be critical. Previously, several studies to elucidate the role of RARs in the differential growth-inhibitory effects of RA in hormone-dependent and -independent breast cancer cell lines were carried out (64, 75). These studies and the present study (Fig. 2) showed comparable expression levels of RAR γ mRNA in all the cell lines, regardless of the ER status, and no marked expression level changes under all-*trans* RA treatment, indicating that RAR γ is unlikely to be involved in the differential growth-inhibitory function of all-*trans* RA. However, a recent study suggests that RAR γ may function to mediate the synergistic growth-inhibitory effect of RA and interferon on breast cancer cells (78). In the case of RAR α , Roman et al. (64) found that it was expressed in all cell lines, with higher levels being found in hormone-dependent cell lines than in independent lines. In our study, two RAR transcripts were observed in all cell lines investigated. However, they were less abundant in MB231 and MB468 cell lines (Fig. 2) but were highly expressed in another hormone-independent line (BT-20). Although these results suggest that RAR α may be involved, the variations in the expression levels of RAR α (reference 64 and this study) cannot satisfactorily explain the dramatic differences in the sensitivities of different breast cancer cell lines to RA. So far, the expression of RAR β has been investigated by several stud-

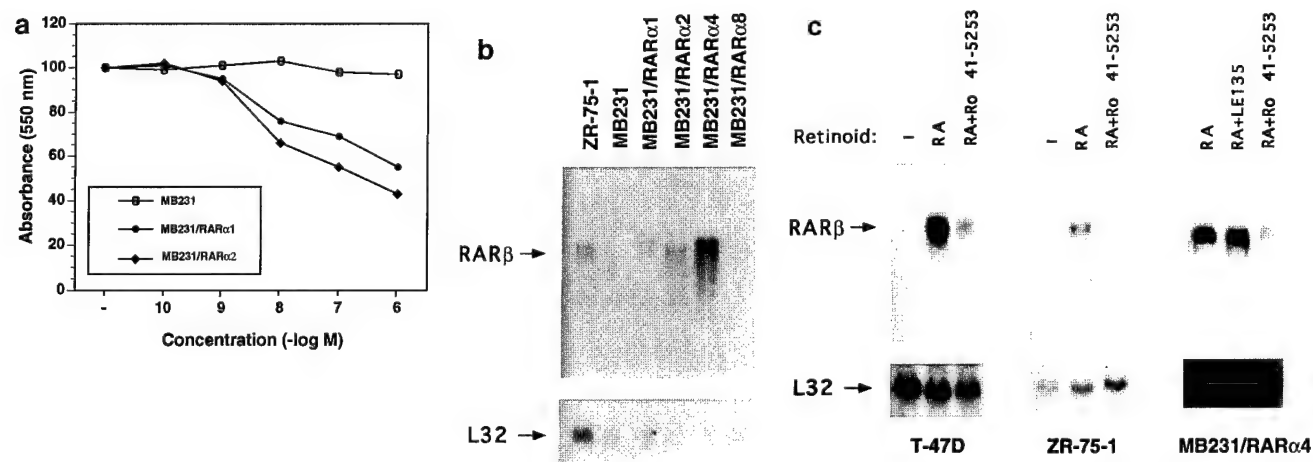


FIG. 6. Inhibition of anchorage-dependent growth of MB231 cells by stable expression of RAR α . (a) The growth of RAR α stable transfectants (MB231/RAR α 1 and MB231/RAR α 2) and parental MB231 cells was analyzed in the presence of various concentrations of all-*trans* RA as described in the legend to Fig. 1. (b) Expression of endogenous RAR β gene in RAR α stable transfectants. Northern blotting was used to analyze the expression of the endogenous RAR β gene in MB231 cells that stably expressed RAR α in the presence of 10^{-6} M all-*trans* RA. For purposes of comparison, the expression of RAR β in ZR-75-1 cells treated with all-*trans* RA is shown. The expression of the L32 gene was used as a control. (c) Inhibition of RA-induced RAR β gene expression by an RAR α -selective antagonist. The expression of the RAR β gene in ZR-75-1 and T-47D cells in the presence of 10^{-7} M all-*trans* RA together with or without 10^{-6} M RAR α -selective antagonist (Ro 41-5253) was analyzed by Northern blotting as described in the legend to Fig. 2. When MB231/RAR α 4 cells were analyzed, 5×10^{-8} M all-*trans* RA and 10^{-7} M Ro 41-5253 and 10^{-7} M LE135 were used.

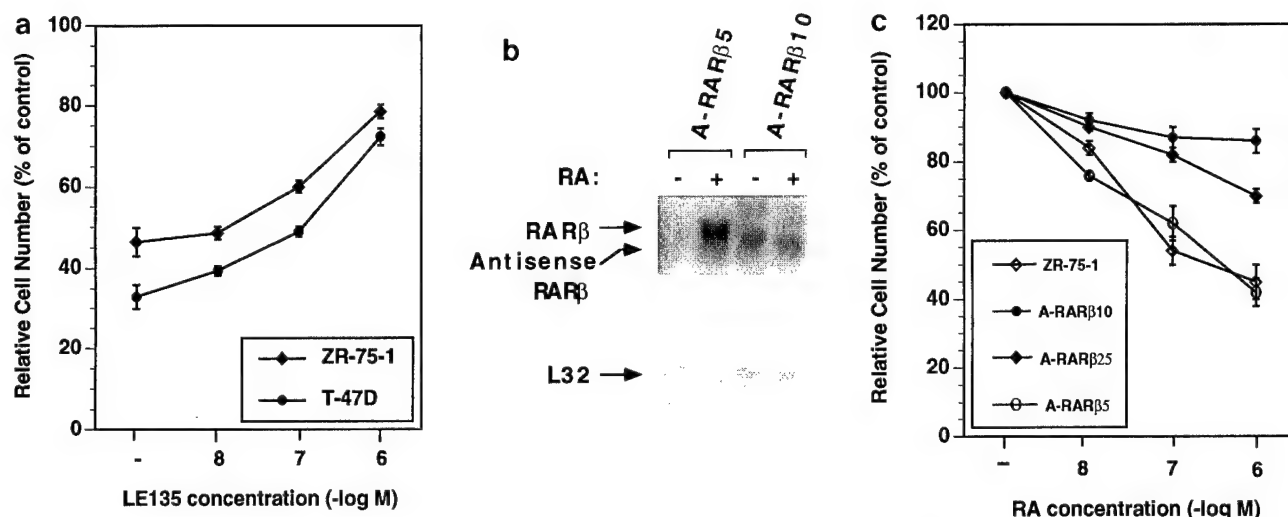


FIG. 7. Inhibition of RAR β activity decreases RA sensitivity in hormone-dependent breast cancer cells. (a) RAR β -selective antagonist decreases the growth-inhibitory effect of all-*trans* RA in ZR-75-1 and T-47D cells. ZR-75-1 and T-47D cells were treated with or without 10^{-7} M all-*trans* RA in the presence or absence of an RAR β -selective antagonist (LE135 K_i for RAR α , 1.5×10^{-6} M; LE135 K_i for RAR β , 4.3×10^{-8} M) for 10 days, and the numbers of the cells were analyzed by the MTT assay as described in the legend to Fig. 1. (b) Expression of RAR β in ZR-75-1 cells transfected with RAR β antisense cDNA. The expression of endogenous RAR β in two stable clones (A-RAR β 5 and A-RAR β 10) was determined by Northern blotting. A-RAR β 10 expressed introduced RAR β antisense RNA, while no clear expression was seen with A-RAR β 5. (c) Expression of RAR β antisense RNA decreases RA sensitivity in hormone-dependent breast cancer cells. The effect of all-*trans* RA on the growth of ZR-75-1 cells and ZR-75-1 cells that stably expressed RAR β antisense RNA (A-RAR β 10 and A-RAR β 25) was analyzed by MTT assay as described in the legend to Fig. 1. Error bars show standard deviations.

ies (64, 72, 75). van der Burg et al. (75) observed high levels of RAR β in two of the three hormone-dependent lines (ZR-75-1 and T-47D) but not in independent lines, except for Hs578T. In contrast, RAR β was expressed in all independent cell lines analyzed by Roman et al. (64), while it was not detected in dependent lines, including T-47D and MCF-7, or was expressed at a low level in other dependent lines. In another study (72), RAR β transcript in MCF-7 and ZR-75-1 cells could only be detected when poly(A)⁺ RNA was used. These different results may be due to the variability of the cell lines used but are more likely to be due to the different culture conditions used, since the expression of RAR β is very sensitive to RA regulation because of the presence of β RARE in its promoter (14, 29, 71). To clearly establish the role of RAR β , we have examined the expression of RAR β in several hormone-dependent and -independent cell lines either in the presence or in the absence of all-*trans* RA (Fig. 2). In the absence of all-*trans* RA, all of these cell lines did not exhibit detectable RAR β transcript. However, in the presence of RA, the expression of RAR β was strongly enhanced in hormone-dependent cell lines (ZR-75-1, T-47D, and MCF-7) but not in independent cell lines (MB231, BT-20, and MB468). The correlation of all-*trans* RA-induced RAR β expression with all-*trans* RA-induced growth inhibition in these cells (Fig. 2) implies that RAR β is required for all-*trans* RA-induced growth inhibition in breast cancer cell lines.

The role of RAR β in mediating all-*trans* RA-induced growth inhibition is further demonstrated by our stable transfection studies. Hormone-independent MB231 cells that are devoid of RAR β did not show a growth response to all-*trans* RA (Fig. 2). However, when the RAR β gene was introduced and expressed in the cells, the inhibitory effects of all-*trans* RA on the anchorage-dependent and -independent growth of the cells were observed (Fig. 4 and 5). In contrast, hormone-dependent ZR-75-1 cells that stably expressed RAR β antisense RNA showed a strong reduced all-*trans* RA sensitivity (Fig. 7). In addition, when we used an RAR β -selective antagonist (LE135) (46) together with all-*trans* RA, we found that it could significantly prevent

all-*trans* RA-induced growth inhibition of ZR-75-1 and T-47D cells (Fig. 7). Together, these data clearly demonstrate that RAR β can mediate the all-*trans* RA-induced growth inhibition in breast cancer cells. This conclusion is supported by a recent study showing that senescence of normal human mammary epithelial cells resulted in increased RAR β mRNA expression (72).

Previous studies (64, 70, 75) and this study (Fig. 2) show that RAR α transcripts are not expressed or are expressed at relatively low levels in certain hormone-independent cell lines, suggesting that RAR α may also participate in mediating the growth inhibition of all-*trans* RA. Indeed, we found that RAR α could restore all-*trans* RA sensitivity when it was stably expressed in MB231 cells (Fig. 6), which is a result similar to that of a previous observation (69). However, RAR α is not responsible for the effect of RAR β , since we did not observe any enhancement of RAR α transcripts in MB231 cells that expressed introduced RAR β (data not shown). When we analyzed the expression of endogenous RAR β in cells that expressed introduced RAR α , we found that it was significantly enhanced in the presence of all-*trans* RA (Fig. 6c). In our transient transfection assay, we observed that cotransfection of RAR α could enhance β RARE activity in hormone-independent cells (unpublished data). Thus, the effect of RAR α may be in part due to its activation of endogenous RAR β through activation of β RARE, which then triggers growth inhibition signaling. However, RAR α alone may not be sufficient to render breast cancer cells responsive to RA, as was seen with BT20 cells, which express a relatively high level of RAR α transcript (Fig. 2a) but which nevertheless are all-*trans* RA resistant (Fig. 2d). It is likely, therefore, that another nuclear protein(s) may participate in the regulation of RA sensitivities in breast cancer cells.

The growth-inhibitory effect of RA appears to be dependent on ER status. Whether this dependence is due to a coincidence resulting from the progression of a tumor or whether estrogen somehow influences RA activities remains to be elucidated. In our stable transfectants expressing RAR β , we did not observe

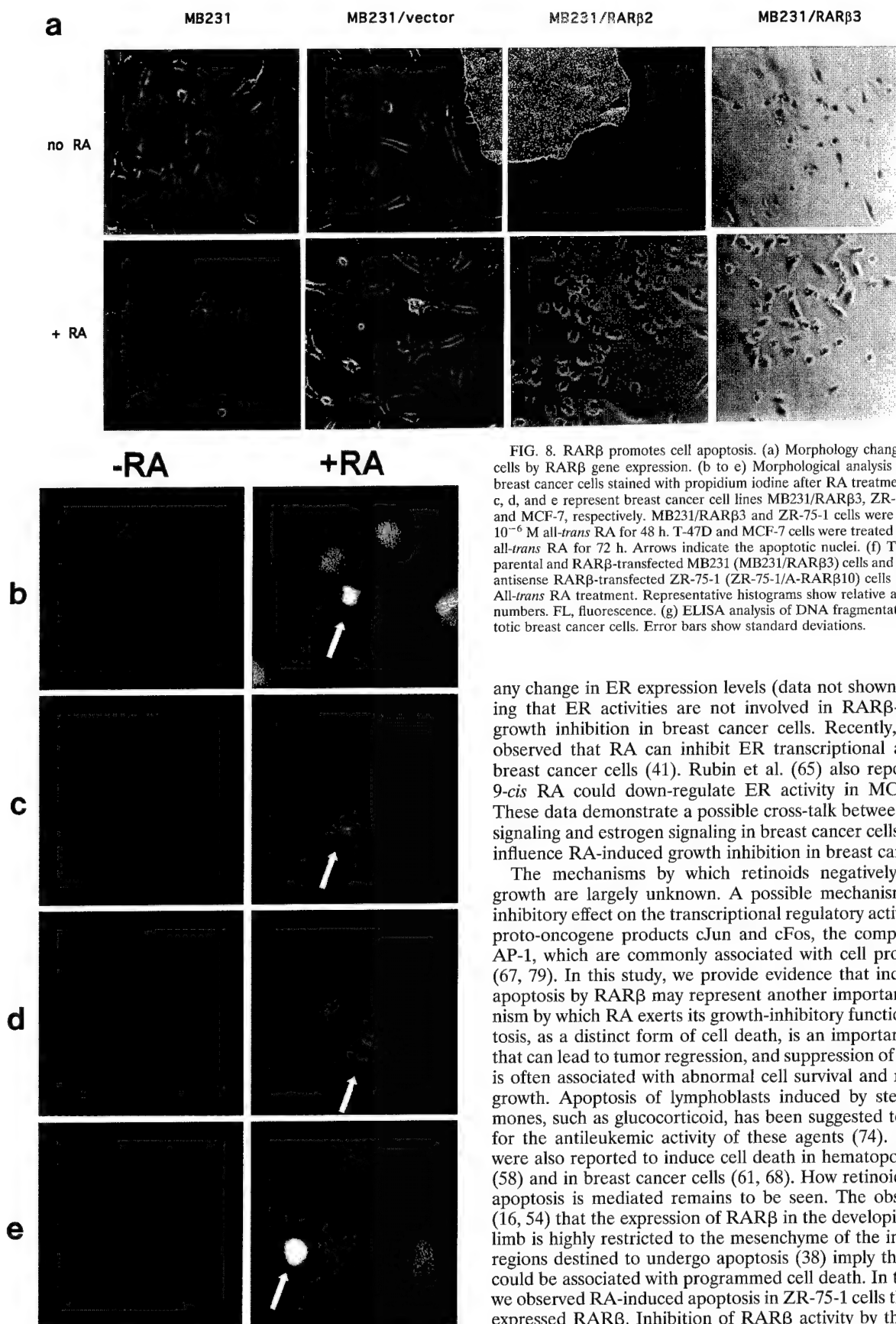
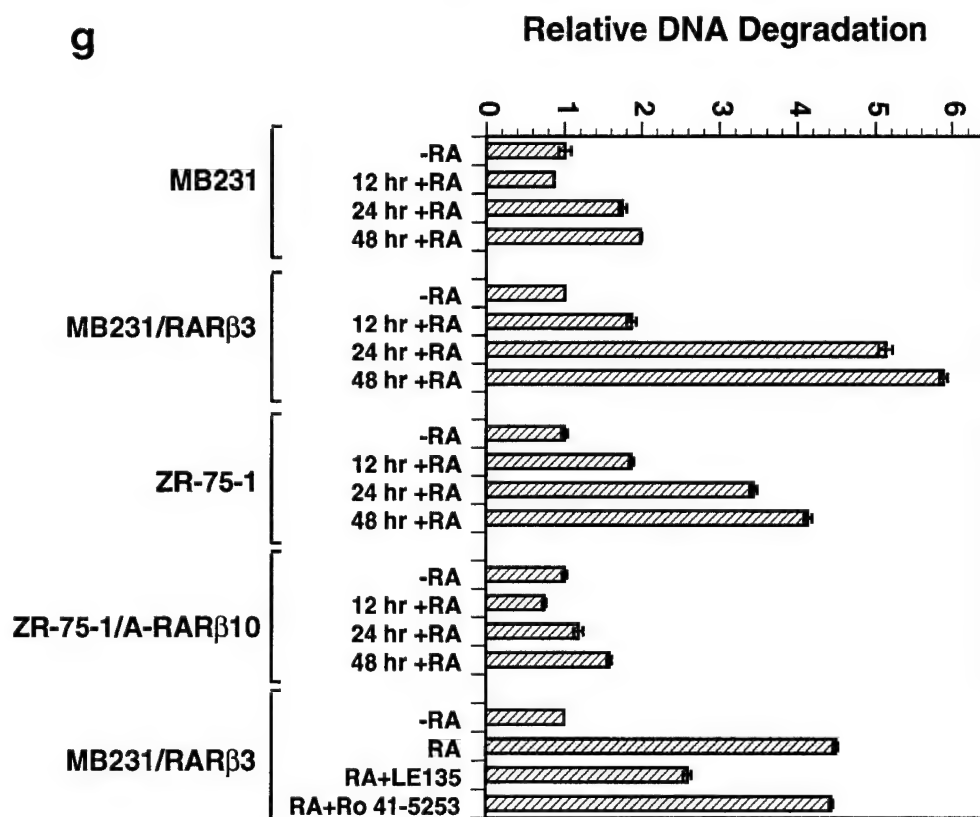
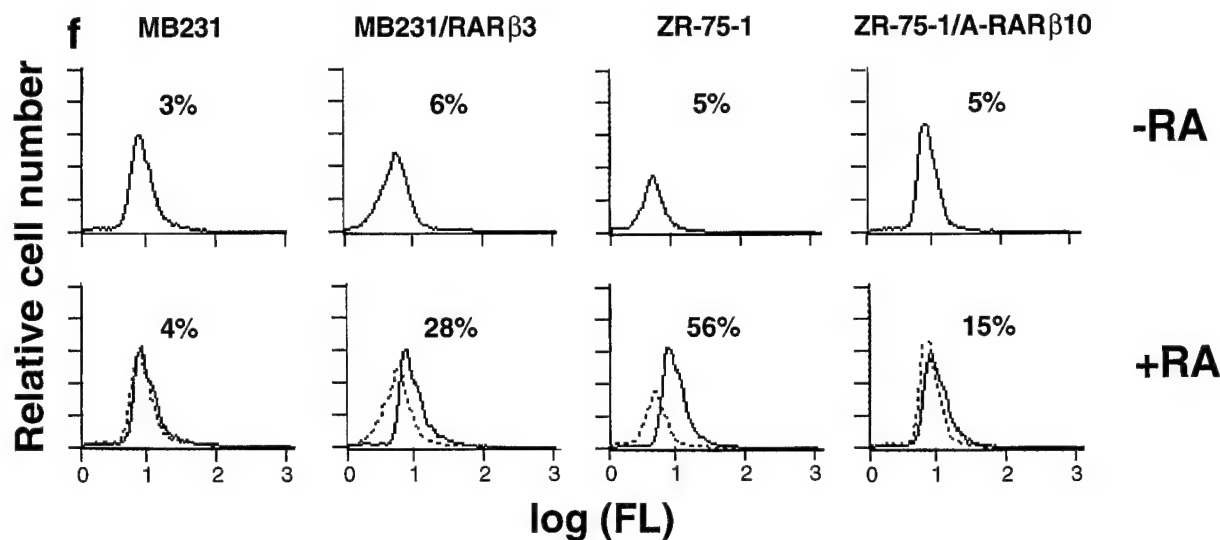


FIG. 8. RAR β promotes cell apoptosis. (a) Morphology change of MB231 cells by RAR β gene expression. (b to e) Morphological analysis of apoptotic breast cancer cells stained with propidium iodide after RA treatment. Panels b, c, d, and e represent breast cancer cell lines MB231/RAR β 3, ZR-75-1, T-47D, and MCF-7, respectively. MB231/RAR β 3 and ZR-75-1 cells were treated with 10^{-6} M all-trans RA for 48 h. T-47D and MCF-7 cells were treated with 10^{-6} M all-trans RA for 72 h. Arrows indicate the apoptotic nuclei. (f) TdT assays of parental and RAR β -transfected MB231 (MB231/RAR β 3) cells and parental and antisense RAR β -transfected ZR-75-1 (ZR-75-1/A-RAR β 10) cells after 24 h of All-trans RA treatment. Representative histograms show relative apoptotic cell numbers. FL, fluorescence. (g) ELISA analysis of DNA fragmentation in apoptotic breast cancer cells. Error bars show standard deviations.

any change in ER expression levels (data not shown), indicating that ER activities are not involved in RAR β -mediated growth inhibition in breast cancer cells. Recently, we have observed that RA can inhibit ER transcriptional activity in breast cancer cells (41). Rubin et al. (65) also reported that 9-*cis* RA could down-regulate ER activity in MCF-7 cells. These data demonstrate a possible cross-talk between retinoid signaling and estrogen signaling in breast cancer cells that may influence RA-induced growth inhibition in breast cancer cells.

The mechanisms by which retinoids negatively regulate growth are largely unknown. A possible mechanism is their inhibitory effect on the transcriptional regulatory activity of the proto-oncogene products cJun and cFos, the components of AP-1, which are commonly associated with cell proliferation (67, 79). In this study, we provide evidence that induction of apoptosis by RAR β may represent another important mechanism by which RA exerts its growth-inhibitory function. Apoptosis, as a distinct form of cell death, is an important process that can lead to tumor regression, and suppression of apoptosis is often associated with abnormal cell survival and malignant growth. Apoptosis of lymphoblasts induced by steroid hormones, such as glucocorticoid, has been suggested to account for the antileukemic activity of these agents (74). Retinoids were also reported to induce cell death in hematopoietic cells (58) and in breast cancer cells (61, 68). How retinoid-induced apoptosis is mediated remains to be seen. The observations (16, 54) that the expression of RAR β in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (38) imply that RAR β could be associated with programmed cell death. In this study, we observed RA-induced apoptosis in ZR-75-1 cells that highly expressed RAR β . Inhibition of RAR β activity by the expres-



sion of RAR β antisense RNA reduced the number of apoptotic cells (Fig. 8g). In contrast, RA-induced apoptosis was only observed in hormone-independent cells when RAR β was introduced and expressed in the cells (Fig. 8g). These data clearly demonstrate that RAR β is able to mediate RA-induced apoptosis of breast cancer cells.

RAR β is expressed in normal breast tissue and in normal human breast cell lines (72). In our study, all-*trans* RA-induced RAR β expression was found only in hormone-dependent breast cancer cell lines and not in independent cell lines. Since hormone-independent breast cancer cells are usually consid-

ered to represent those at a late stage of breast tumor progression, this observation suggests that the loss of RAR β gene expression may have important pathogenic consequences during the development of human breast cancer. Alteration of RAR β activities may be involved in the development of human liver cancer (12). In lung and breast cancer, a deletion of the short arm of chromosome 3p, a region that maps close to the RAR β gene, occurs with high frequency (15, 59, 80). Abnormally low levels of RAR β gene expression were observed in many human lung cancer cell lines and other cancer cell lines (22, 30, 32, 60, 86) and were suggested to contribute to the

neoplastic progression of human oral squamous cell carcinoma cell lines (32) and the tumorigenicity of human papillomavirus type 18-transformed HeLa cells (3). Furthermore, RAR β has been shown to function as a tumor suppressor gene in epidermoid lung carcinoma cells (31). These observations, therefore, suggest that a low expression level of the RAR β gene may be an important contributing factor for cancer development. The present finding that RAR β can promote apoptosis suggests that the loss of RAR β activities could remove such a negative control mechanism, resulting in uncontrolled cell proliferation and, therefore, an enhancement of the transformed phenotype of the cells.

In conclusion, our results demonstrate that the loss of RAR β gene expression and regulation by all-*trans* RA is a common feature associated with hormone-independent breast cancer cells and may be one of the major factors responsible for diminished retinoid sensitivity during the progression of a breast tumor. Thus, the expression level of the RAR β gene and its response to RA may serve as diagnostic factors for cancer and may be also used to determine whether patients with breast cancer will respond to RA treatment. Our results also demonstrate that RAR β mediates the growth-inhibitory effect of RA in part by inducing cell apoptosis, which, when lost, may contribute to cancer development. The observation that introduction of the RAR β gene into RAR β -negative cancer cells can restore RA sensitivities provides valuable directions for the development of new strategies for the treatment of human breast cancer.

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ADDENDUM

Seewaldt et al. have recently reported that RAR β can mediate growth arrest and induce apoptosis in human breast cancer cells (67a); their results support our discovery in this study.

REFERENCES

- Alam, M., V. Zhestkov, B. P. Sani, P. Venepally, A. A. Levin, S. Kazmer, E. Li, A. W. Norris, X.-K. Zhang, M.-O. Lee, D. L. Hill, T.-H. Lin, W. J. Brouillette, and D. D. Muccio. 1995. Conformationally defined 6-s-*trans*-retinoic acid analogs. 2. Selective agonists for nuclear receptor binding and transcriptional activity. *J. Med. Chem.* **38**:2302-2310.
- Apfel, C., F. Bauer, M. Crettaz, L. Forni, M. Kamber, F. Kaufmann, P. LeMotte, W. Pirson, and M. Klaus. 1992. A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. *Proc. Natl. Acad. Sci. USA* **89**:7129-7133.
- Bartsch, D., B. Boye, C. Baust, H. Z. Hausen, and E. Schwarz. 1992. Retinoic acid-mediated repression of human papillomavirus 18 transcription and different ligand regulation of the retinoic acid receptor β gene in non-tumorigenic and tumorigenic HeLa hybrid cells. *EMBO J.* **11**:2283-2291.
- Benbrook, D., E. Lernhardt, and M. Pfahl. 1988. A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature (London)* **333**:624-629.
- Boccardo, F., L. Canobbio, M. Resasco, A. U. Decensi, G. Pastorino, and F. Brema. 1990. Phase II study of tamoxifen and high-dose retinyl acetate in patients with advanced breast cancer. *J. Cancer Res. Clin. Oncol.* **116**:503-506.
- Brand, N., M. Petkovich, A. Krust, H. de The, A. Marchio, P. Tiollais, and A. Dejean. 1988. Identification of a second human retinoic acid receptor. *Nature (London)* **332**:850-853.
- Bugge, T. H., J. Pohl, O. Lonnoy, and H. J. Stunnenberg. 1992. RXR α , a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J.* **11**:1409-1418.
- Bunch, T. A., and L. S. B. Goldstein. 1989. The conditional inhibition of gene expression in cultured *Drosophila* cells by antisense RNA. *Nucleic Acids Res.* **17**:9761-9782.
- Cassidy, J., M. Lippman, A. LaCroix, and G. Peck. 1982. Phase II trial of 13-*cis*-retinoic acid in metastatic breast cancer. *Eur. J. Cancer Clin. Oncol.* **18**:925-928.
- Chambon, P. 1994. The retinoid signaling pathway: molecular and genetic analyses. *Semin. Cell Biol.* **5**:115-125.
- Costa, A. 1993. Breast cancer chemoprevention. *Eur. J. Cancer* **29A**:589-592.
- Dejean, A., L. Bougueleret, K.-H. Grzeschik, and P. Tiollais. 1986. Hepatitis B virus DNA integration in a sequence homologous to c-erb-A and steroid receptor genes in a hepatocellular carcinoma. *Nature (London)* **322**:70-72.
- de The, H., C. Lavau, A. Marchio, C. Chomienne, L. Degos, and A. Dejean. 1991. The PML-RAR α fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* **66**:675-684.
- de The, H., M. D. M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, and A. Dejean. 1990. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature (London)* **343**:177-180.
- Devilee, P., M. van Vliet, P. van Sloun, N. K. Dijkshoorn, J. Hermans, P. L. Pearson, and C. J. Cornelisse. 1991. Allelotype of human breast carcinoma: a second major site for loss of heterozygosity is on chromosome 6q. *Oncogene* **6**:1705-1711.
- Dolle, P., J. C. Izpisua-Belmonte, H. Falkenstein, A. Renucci, and D. Duboule. 1989. Differential expression of genes encoding α , β , γ retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature (London)* **342**:702-705.
- Evans, R. M. 1988. The steroid and thyroid hormone receptor family. *Science* **240**:889-895.
- Eyrolles, L., H. Kagechika, E. Kawachi, H. Fukasawa, T. Iijima, Y. Matsushima, Y. Hashimoto, and K. Shudo. 1994. Retinobenzoic acids. 6. Retinoid antagonists with a heterocyclic ring. *J. Med. Chem.* **37**:1508-1517.
- Fisher, D. E. 1994. Apoptosis in cancer therapy: crossing the threshold. *Cell* **78**:539-542.
- Fontana, J. A. 1987. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation. *Exp. Cell Biol.* **55**:136-144.
- Fontana, J. A., P. D. Hobbs, and M. I. Dawson. 1988. Inhibition of mammary carcinoma growth by retinoid benzoic acid derivatives. *Exp. Cell Biol.* **56**:254-263.
- Gebert, J. F., N. Moghal, J. V. Frangioni, D. J. Sugarbaker, and B. G. Neel. 1991. High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* **6**:1859-1868.
- Giguere, V., E. S. Ong, P. Seigi, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. *Nature (London)* **330**:624-629.
- Gorczyca, W., J. Gong, and Z. Darzynkiewicz. 1993. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* **53**:1945-1951.
- Green, S., and P. Chambon. 1988. Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet.* **4**:309-314.
- Gudas, L. J., M. B. Sporn, and A. B. Roberts. 1994. Cellular biology and biochemistry of the retinoids, p. 443-520. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*, 2nd ed. Raven Press, New York.
- Hamada, K., S. L. Gleason, B. Z. Levi, S. Hirschfeld, E. Appella, and K. Ozato. 1989. H-2RIIBP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. *Proc. Natl. Acad. Sci. USA* **86**:8289-8293.
- Heyman, R., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller. 1992. 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **57**:397-406.
- Hoffmann, B., J. M. Lehmann, X.-K. Zhang, T. Hermann, G. Graupner, and M. Pfahl. 1990. A retinoic acid receptor-specific element controls the retinoic acid receptor- β promoter. *Mol. Endocrinol.* **4**:1734-1743.
- Houle, B., F. Ledue, and W. E. C. Bradley. 1991. Implication of RAR β in epidermoid (squamous) carcinoma cells. *Exp. Cell Res.* **195**:163-170.
- Houle, B., C. Rochette-Egly, and W. E. C. Bradley. 1993. Tumor-suppressive effect of the retinoic acid receptor β in human epidermoid lung cancer cells. *Proc. Natl. Acad. Sci. USA* **90**:985-989.
- Hu, L., D. L. Crowe, J. G. Rheinwald, P. Chambon, and L. Gudas. 1991. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.* **51**:3972-3981.
- Hunter, D. J., J. E. Manson, G. A. Colditz, M. J. Stampfer, B. Rosner, C. H. Hennekens, F. E. Speizer, and W. C. Willett. 1993. A prospective study of the intake of vitamins C, E, and A and the risk of breast cancer. *N. Engl. J. Med.* **329**:234-240.
- Jacobson, M. D., J. F. Burne, M. P. King, T. Miyashita, J. C. Reed, and M. C. Raff. 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature (London)* **361**:365-369.
- Kagechika, H., E. Kawachi, Y. Hashimoto, and K. Shudo. 1989. Retinobenzoic acids. 2. Structure-activity relationships of chalcone-4-carboxylic acids and flavone-4'-carboxylic acids. *J. Med. Chem.* **32**:834-840.
- Kakizuka, A., W. H. Miller, Jr., K. Umehono, R. P. Warrell, Jr., S. R. Frankel, V. V. S. Murty, E. Dmitrovsky, and R. M. Evans. 1991. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcriptional factor, PML. *Cell* **66**:663-674.
- Kliwer, S. A., K. Umehono, D. J. Mangelsdorf, and R. M. Evans. 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signaling. *Nature (London)* **355**:446-449.

38. Kochhar, D. M., H. Jiang, D. C. Harnish, and D. R. Soprano. 1993. Evidence that retinoic acid-induced apoptosis in the mouse limb bud core mesenchymal cells is gene-mediated. *Prog. Clin. Biol. Res.* **383B**:815-825.
39. Koga, M., and R. L. Sutherland. 1991. Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D3 or antiestrogen to inhibit human breast cancer cell proliferation. *J. Steroid Biochem. Mol. Biol.* **39**:455-460.
40. Krust, A., P. H. Kastner, M. Petkovich, A. Zelent, and P. Chambon. 1989. A third human retinoic acid receptor, hRAR γ . *Proc. Natl. Acad. Sci. USA* **86**:5310-5314.
41. Lee, M.-O., Y. Liu, and X.-K. Zhang. 1995. A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. *Mol. Cell. Biol.* **15**:4194-4207.
42. Lehmann, J. M., X.-K. Zhang, and M. Pfahl. 1992. RAR γ 2 expression is regulated through a retinoic acid response element embedded in Sp1 sites. *Mol. Cell. Biol.* **12**:2976-2985.
43. Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J. M. Garnier, S. Mader, and P. Chambon. 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**:377-395.
44. Leroy, P., H. Nakshatri, and P. Chambon. 1991. Mouse retinoic acid receptor α 2 isoform is transcribed from a promoter that contains a retinoic acid response element. *Proc. Natl. Acad. Sci. USA* **88**:10138-10142.
45. Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzelisen, M. Rosenberger, A. Lovey, and J. F. Grippo. 1992. 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR α . *Nature (London)* **355**:359-361.
46. Li, Y., Y. Hashimoto, and X.-K. Zhang. Unpublished data.
47. Lippman, S. M., J. F. Kessler, and F. L. Meyskens. 1987. Retinoids as preventive and therapeutic anticancer agents. *Cancer Treat. Rep.* **71**:493-515.
48. Lotan, R. 1981. Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* **605**:33-91.
49. Mangelsdorf, D. J., U. Borgmeyer, R. A. Heyman, J. Y. Zhou, E. S. Ong, A. E. Oro, A. Kakizuka, and R. M. Evans. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6**:329-344.
50. Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature (London)* **345**:224-229.
51. Mangelsdorf, D. J., K. Umehono, and R. M. Evans. 1994. The retinoid receptors, p. 319-349. In M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*, 2nd ed. Raven Press, New York.
52. Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Apella, V. M. Nikodem, and K. Ozato. 1992. H-2R1BP (RXR β) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J.* **11**:1419-1435.
53. Marth, C., G. Bock, and G. Daxenbichler. 1985. Effect of 4-hydroxyphenyl-retinamide and retinoic acid on proliferation and cell cycle of cultured human breast cancer cells. *JNCI* **75**:871-875.
54. Mendelsohn, C., E. Ruberte, M. LeMeur, G. Morris-Kay, and P. Chambon. 1991. Developmental analysis of the retinoic acid-inducible RAR β promoter in transgenic animals. *Development (Cambridge)* **113**:723-734.
55. Modiano, M., W. Dalton, S. Lippman, L. Joffe, A. R. Booth, and F. L. Meyskens, Jr. 1990. Phase II study of fenretinide (n-[4-hydroxyphenyl]retinamide) in advanced breast cancer and melanoma. *Invest. New Drugs* **8**:317-319.
56. Moon, R. C., and R. G. Mehta. 1990. Chemoprevention of mammary cancer by retinoids. *Basic Life Sci.* **52**:213-224.
57. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55-63.
58. Nagy, L., V. A. Thomázy, G. L. Shipley, L. Fésüs, W. Lamph, R. A. Heyman, R. A. S. Chandraratna, and P. J. A. Davies. 1995. Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. *Mol. Cell. Biol.* **15**:3540-3551.
59. Naylor, S. L., B. E. Johanson, J. D. Minna, and A. Y. Sakaguchi. 1987. Loss of heterozygosity of chromosome 3p markers in small cell lung cancer. *Nature (London)* **329**:451-453.
60. Nervi, C., T. M. Volleberg, M. D. George, A. Zelent, P. Chambon, and A. M. Jetten. 1991. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp. Cell Res.* **195**:163-170.
61. Pellegrini, R., A. Mariotti, E. Tagliabue, R. Bressan, G. Bunone, D. Coradini, G. Della Valle, F. Formelli, L. Cleris, P. Radice, M. A. Pierotti, M. I. Colnaghi, and S. Menard. 1995. Modulation of markers associated with tumor aggressiveness in human breast cancer cell lines by N-(4-hydroxyphenyl)retinamide. *Cell Growth Differ.* **6**:863-869.
62. Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature (London)* **330**:444-450.
63. Roberts, A. B., and M. B. Sporn. 1984. Cellular biology and biochemistry of the retinoids, p. 209-286. In M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*, vol. 2. Academic Press, Orlando, Fla.
64. Roman, S. D., C. L. Clarke, R. E. Hall, I. E. Alexander, and R. L. Sutherland. 1992. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.* **52**:2236-2242.
65. Rubin, M., E. Fenig, A. Rosenauer, C. Menendez-Botet, C. Achkar, J. M. Bentel, J. Yahalom, J. Mendelsohn, and W. H. Miller, Jr. 1994. 9-cis retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein. *Cancer Res.* **54**:6549-6556.
66. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
67. Schule, R., P. Rangarajan, N. Yang, S. Kliewer, L. J. Ransone, J. Bolado, I. M. Verma, and R. M. Evans. 1991. Retinoic acid is a negative regulator of AP-1 responsive genes. *Proc. Natl. Acad. Sci. USA* **88**:6092-6096.
- 67a. Seewaldt, V. L., B. S. Johnson, M. B. Parker, S. J. Collins, and K. Swisselshelm. 1995. Expression of retinoic acid receptor β mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. *Cell Growth Differ.* **6**:1077-1088.
68. Shao, Z. M., M. I. Dawson, X. S. Li, A. K. Rishi, Q. X. Han, J. V. Ordenez, B. Shroot, and J. A. Fontana. 1995. p53 independent G0/G1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells. *Oncogene* **11**:493-504.
69. Sheikh, M. S., A.-M. Shao, X.-S. Li, M. Dawson, A. M. Jetten, S. Wu, B. A. Conley, M. Garcia, H. Rochefort, and J. A. Fontana. 1994. Retinoid-resistant estrogen receptor-negative human carcinoma cells transfected with retinoic acid receptor- α acquire sensitivity to growth inhibition by retinoids. *J. Biol. Chem.* **269**:21440-21447.
70. Sheikh, M. S., Z.-M. Shao, J.-C. Chen, A. Hussain, A. M. Jetten, and J. A. Fontana. 1993. Estrogen receptor-negative breast cancer cells transfected with the estrogen receptor exhibit increased RAR α gene expression and sensitivity to growth inhibition by retinoic acid. *J. Cell. Biochem.* **53**:394-404.
71. Sucov, H. M., K. K. Murakami, and R. M. Evans. 1990. Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc. Natl. Acad. Sci. USA* **87**:5392-5396.
72. Swisselshelm, K., K. Ryan, X. Lee, H. C. Tsou, M. Beacocke, and R. Sager. 1994. Down-regulation of retinoic acid receptor β in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.* **5**:133-141.
73. Teilmann, K., T. Tsukaguchi, M. Klaus, and J. F. Eliason. 1993. Comparison of the therapeutic effects of a new arotinoid, Ro 40-8757, and all-trans- and 13-cis-retinoic acid on rat breast cancer. *Cancer Res.* **53**:2319-2325.
74. Thompson, E. B. 1994. Apoptosis and steroid hormones. *Mol. Endocrinol.* **8**:665-673.
75. van der Burg, B., B.-J. M. van der Leede, L. Kwakkenbox-Isbrucker, S. Salverda, S. W. de Laat, and P. T. van der Saag. 1993. Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol. Cell. Endocrinol.* **91**:149-157.
76. van der Leede, B. M., C. E. van den Brink, and P. T. van der Saag. 1993. Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines. *Mol. Carcinog.* **8**:112-122.
77. Wetherall, N. T., and C. M. Taylor. 1986. The effects of retinoid treatment and antiestrogens on the growth of T47D human breast cancer cells. *Eur. J. Cancer Clin. Oncol.* **22**:53-59.
78. Widschwendter, M., G. Daxenbichler, O. Dapunt, and C. Marth. 1995. Effects of retinoic acid and gamma-interferon on expression of retinoic acid receptor and cellular retinoic acid-binding protein in breast cancer cells. *Cancer Res.* **55**:2135-2139.
79. Yang-Yen, H. F., X.-K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, and M. Pfahl. 1991. Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation. *New Biol.* **3**:1206-1219.
80. Yokota, J., M. Wada, Y. Shimosato, M. Terada, and T. Sugimura. 1987. Loss of heterozygosity on chromosome 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. USA* **84**:9252-9256.
81. Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Naar, S. Y. Kim, J.-M. Boutin, C. K. Glass, and M. G. Rosenfeld. 1991. RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**:1251-1266.
82. Zhang, X.-K., B. Hoffmann, P. Tran, G. Graupner, and M. Pfahl. 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature (London)* **355**:441-446.
83. Zhang, X.-K., J. Lehmann, B. Hoffmann, M. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl. 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature (London)* **358**:587-591.
84. Zhang, X.-K., Y. Liu, M.-O. Lee, and M. Pfahl. 1994. A specific defect in the retinoic acid receptor associated with human lung cancer cell lines. *Cancer Res.* **54**:5663-5669.
85. Zhang, X.-K., and M. Pfahl. 1993. Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.* **4**:156-162.
86. Zhang, X.-K., G. Salbert, M.-O. Lee, and M. Pfahl. 1994. Mutations that alter ligand-induced switches and dimerization activities in the retinoid X receptor. *Mol. Cell. Biol.* **14**:4311-4323.

Retinoid receptors in human lung cancer and breast cancer

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Abstract

Retinoids, the natural and synthetic vitamin A derivatives, are known to inhibit the proliferation of lung cancer and breast cancer cells and the growth of carcinogen-induced bronchogenic squamous cell carcinoma and mammary tumors, and have been used as chemoprevention agents against both types of cancer. However, clinical trials of retinoids in patients with advanced lung cancer and breast cancer have not been successful. In studying how retinoid sensitivity is lost in cancer cells, we have found that lack of the retinoic acid receptor β (RAR β) gene expression and its abnormal regulation by retinoic acid (RA) are common features in human lung cancer and breast cancer cells. The absence and abnormal RA regulation of RAR β correlates with the loss of anti-proliferation effect of RA in hormone-independent breast cancer cells, and is due to different abnormalities found in cancer cells. Furthermore, expression of RAR β gene in hormone-independent breast cancer cells restores their RA sensitivity. These data demonstrate that RAR β can mediate the growth inhibitory effect of RA and suggest that the lack of RAR β may contribute to retinoid resistance in certain cancer cells.

Keywords: Chemoprevention; Retinoic acid receptor β ; Retinoic acid; Retinoid resistance; Malignancy; Carcinogenesis

1. Background

Retinoids are a group of natural and synthetic vitamin A analogs that exert profound effects on the growth and differentiation of normal, premalignant and malignant epithelial cells in vitro and in vivo [1,2]. They have been found to suppress carcinogenesis in experimental animals and exhibit different degrees of efficacy in chemoprevention and therapy of many types of malignancy [1–3]. The exciting results of several clinical trials concerning the beneficial effect of retinoids have been recently reported.

Retinoic acid (RA) showed dramatic anti-tumor effects in patients with acute promyelocytic leukemia (APL) [4–7] and high response rates for patients with cervical cancer [8] and metastatic squamous cancer of the skin [9]. Isotretinoin (13-*cis*-retinoic acid) was reported to cause repression of premalignant leukoplakia of the buccal mucosa [10] and can reverse premalignant lesions and prevent the occurrence of second primary tumors in patients with squamous-cell carcinoma of the head and neck [11].

Since retinoids are involved in the general maintenance or enhancement of differentiation and cancer is a process in which loss of differentiation occurs, retinoids have been considered as potent cancer chemopreventive agents. So far, the preventive effect of retinoids against lung cancer or breast cancer has

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been well recognized. Low dietary intake or serum levels of β -carotene are often associated with increased risk of lung cancer and breast cancer [12-15]. Vitamin A can inhibit the carcinogenesis processes of chemical-induced bronchogenic squamous cell carcinoma and anaplastic carcinoma as well as mammary tumor [16-19], and its deficiency induces squamous metaplasia, a commonly found premalignant lesion [20]. Similarly, retinoids can affect regression of premalignant lung lesions and convert metaplastic bronchi in heavy cigarette smokers to normal [21]. In vitro, RA plays a critical role in regulating the differentiation and proliferation of tracheobronchial epithelial cells and is known to suppress the squamous differentiation of these cells [20,22,23]. Promising results have been recently reported using 13-*cis*-RA in chemoprevention trials in lung and upper-aerodigestive tract cancer, showing that this retinoid can reverse premalignant lesions and prevent the occurrence of second primary tumors [24]. In breast cancer cells, retinoids are effective inhibitors of their proliferation [25-30]. However, inhibition on growth of breast cancer cells in culture was often observed in hormone-dependent but not in hormone-independent breast cancer cells. The inhibition was seen when retinoids are administered alone or in combination with anti-estrogen [31-34], where synergistic effects were found. In addition, the synergistic effects in inhibiting the initiation and promotion of mammary tumors induced by carcinogens were reported when retinoids was used in combination with ovariectomy or anti-estrogens [18,19]. These observations indicate that retinoids can act as effective cancer chemopreventive agents and may serve as potential chemotherapeutic agents against lung cancer and breast cancer. However, clinical trials of retinoids in patients with advanced lung cancer or breast cancer have not demonstrated significant activities [3,24,35-38], suggesting possible modification of retinoid response during progression of these tumors.

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) [39-44] and the retinoid X receptors (RXRs) [45-49]. Both types of receptors are encoded by three distinct genes, α , β and γ [39-49]. RARs and RXRs are part of the steroid/thyroid hormone receptor superfamily [50,51]. They function as ligand-

activated transcription factors that bind to specific RA responsive sequences (RARE) on the target genes as RXR/RAR or RXR/RXR dimeric complexes ([47,48,52-56]) and regulate the transcriptional expression of these genes. One of the target genes is RAR β itself and a RARE (β RARE) has been identified in its promoter region, that consists of two directly repeated copies of the core motif sequence (G/A)GTTCA separated by 5 nucleotides [57-59]. The level of RAR β transcript increases dramatically in response to RA due the presence of β RARE [57-59]. This auto-induction of RAR β transcription was observed in many different cell types and tissues including lung and mammary tissues [60,61].

Despite extensive research on the mechanism by which RA functions, how retinoids inhibit the proliferation of cancer cells and how retinoid sensitivity is lost in advanced tumors are unclear. Since the retinoid responses are mainly mediated by their nuclear receptors, we have investigated the expression of retinoid receptor genes (RARs and RXRs) in different human lung and breast cancer cell lines. Our results reveal a high frequency of undetectable expression and abnormal RA regulation of RAR β gene in human lung cancer and breast cancer cell lines and suggest that the lack of the RAR β gene expression and its responsiveness to RA may represent a general alteration of RA sensitivity program in cancer cells, which may contribute to cancer development and may account for the ineffective treatment in using retinoids for patients with advanced tumor.

2. Abnormal expression and RA regulation of the RAR β gene in human lung cancer cell lines

As the first step to establish the involvement of RARs and RXRs in mediating retinoid response in lung cancer cells and whether altered expression and function of these receptor genes contributes to impaired RA response in the cells, we investigated the expression of these genes in various lung cancer cell lines, including small cell lung carcinoma (H146), squamous cell carcinoma (SK-MES-1), adenosquamous carcinoma (H292), large cell carcinoma (H661), adenocarcinoma (Calu-3) and anaplastic carcinoma (Calu-6). Similar expression levels of RAR α and RAR γ as well as RXR α and RXR β genes

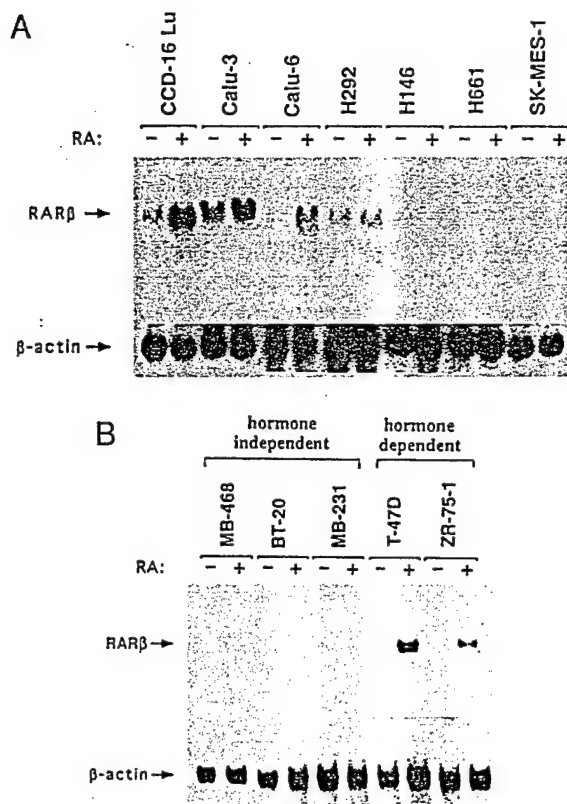


Fig. 1. The expression of RAR β gene in the human lung cancer (A) and breast cancer (B) cell lines. The expression of RAR β gene was determined by Northern-blot analysis using total RNA prepared from different lung cancer (A) and breast cancer (B) cell lines. Total RNAs from lung cancer cell lines (20 μ g) and from breast cancer cell lines (30 μ g) were separated, transferred to Nylon filters, and probed with the 32 P-labeled ligand-binding domain of RAR β . To determine the effect of RA, cells were treated with 10^{-6} M RA for 36 h before RNA preparation. The expression of the β -actin gene was used as a control to ensure that same amounts of RNA were used.

were detected in all these lines [62]. However, the expression levels of RAR β gene varied dramatically among them (Fig. 1A). It was highly expressed in Calu-3 and H292 cells while its expression was not detectable in Calu-6, H146, H661 and SK-MES-1 cells. Thus, the RAR β gene may be abnormally expressed in the majority of human lung cancer cell lines. This result is consistent with observation made by other groups when other lung cancer cell lines were studied [63–66], suggesting that lack of the RAR β gene expression may be involved in the development of lung cancer.

RAR β gene is a well known RA-responsive gene due to the presence of β RARE in its promoter [57–59]. The β RARE is a potent RA response element, which can be activated by RAR/RXR heterodimers [52] in many different cell types, including F9 and P19 embryonal carcinoma cells. In normal lung tissue, a 16-fold induction of RAR β transcript by RA was observed [61]. Thus, the expression of the RAR β gene in response to RA may be used to determine the degree of general RA responsiveness in cells. To study RA response, we examined the effect of RA in inducing RAR β gene expression in different human lung cancer cell lines (Fig. 1A). In contrast to the dramatic effect of RA in inducing RAR β gene expression in normal lung and many other cell lines [57–61], addition of RA did not induce RAR β expression in most of the lung cancer cell lines investigated (Fig. 1A). When the cells were exposed to RA, the level of RAR β transcript in normal lung 16-Lu cells was dramatically enhanced (about 10-fold). Similarly, the expression of RAR β gene was enhanced in Calu-6 cells. In contrast, treatment with RA failed to induce the expression of RAR β gene in H146, H661 and SK-MES-1 cells. Although H292 and Calu-3 cells expressed high levels of the RAR β transcript, treatment of the cells did not influence their expression levels. To ensure that an equal amounts of RNA were used for each sample, the expression of β -actin gene was examined for comparison. Thus, the low expression levels and the loss of the RA sensitivity in inducing RAR β gene expression are common features of these cancer cells.

3. Anti-proliferation effect of RA and RAR β gene expression in human breast cancer cell lines

Retinoids are effective inhibitors of the proliferation of breast cancer cells [25–30]. However, despite extensive research on retinoid response in breast cancer cells, how the growth inhibitory effects of retinoids are lost in hormone-independent breast cancer cells is presently unclear. We examined the expression of the retinoid receptor genes in a number of hormone-dependent (ZR-75-1 and T-47D) and hormone-independent (MB468, MB231, BT-20) cell lines. Similar expression levels of the RAR α , RAR γ ,

RXR α and RXR β were detected in these cell lines (data not shown). However, the expression of the RAR β gene was not seen in all these lines (Fig. 1B). When the effect of RA on the expression of the RAR β gene was analyzed, we observed a strong induction of the RAR β gene expression in two hormone-dependent cell lines. Interestingly, the induction was not seen in all hormone-independent cell lines (Fig. 1B). The loss of RA response in inducing RAR β gene expression in all the hormone-independent human breast cancer cell lines investigated indicates that the regulation of the RAR β gene expres-

sion by RA is disturbed in these cell lines. Recently, it was also demonstrated that the expression of the RAR α gene is relatively lower in some hormone-independent breast cancer cell lines [67-69]. Since hormone-independent breast cancer cells are usually considered to represent those at late stage of breast tumor progression, these observations suggest that the lack of RARs may have important pathogenic consequence during the development of human breast cancer and may be responsible for the diminishment of retinoid sensitivity during the progression of breast tumor.

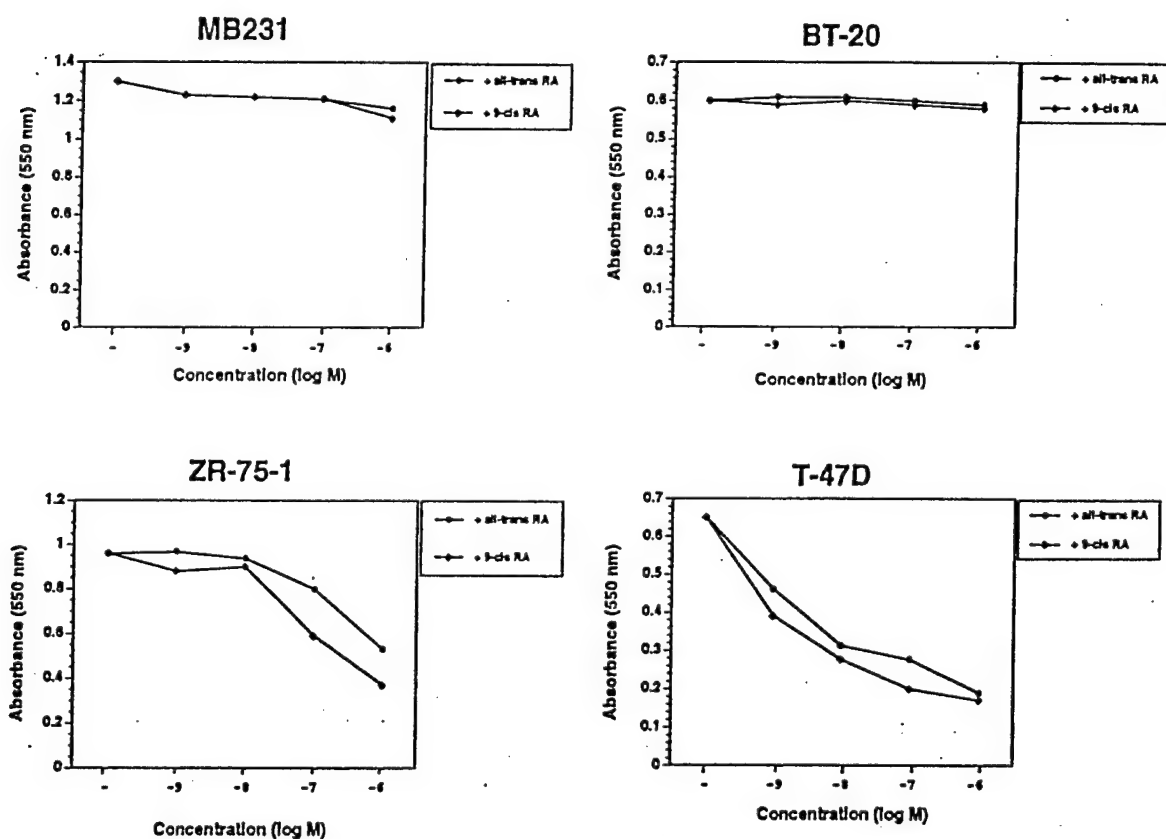


Fig. 2. Effect of all-trans RA and 9-cis RA on the growth of hormone-dependent and -independent breast cancer cells. To investigate whether all-trans RA and 9-cis RA could inhibit the proliferation of human breast cancer cells, the effect of RAs on the growth of hormone-dependent (ZR75-1 and T-47D) and -independent (MB231 and BT-20) breast cancer cells was analyzed using a non-radioactive Cell Proliferation/Cytotoxicity Assay Kit (Promega). Briefly, cells were seeded at 1000-2000 cells per well in 96-well plates (Costar), and treated with various concentrations of all-trans RA or 9-cis RA for 7 days. Media were changed every 48 h. The number of viable cells were determined by their capacity to convert a tetrazolium salt into a blue formazan product that was measured using an ELISA plate reader. The results were expressed as a percentage of absorbance at 550 nm of MTT-derived formazan developed by cells treated with control solvent.

4. RAR β mediates the anti-proliferation effect of RA in human breast cancer cells

The fact that RAR β is not expressed in many lung cancer and breast cancer cell lines points to the possibility that it may mediate the anti-growth effect of retinoids and that the lack of RAR β may contribute to retinoid resistance in these cancer cells. To determine whether the expression levels of RAR β could reflect biological response of breast cancer cells to retinoids, we measured the growth inhibitory effect of all-*trans* RA and 9-*cis*-RA on several breast cancer cell lines by MTT assay (Fig. 2). Both RAs showed a strong inhibition of the growth of hormone-dependent cell lines (T47D and ZR-75-1) while they had little effect on hormone-independent lines. Thus, the induction of RAR β gene expression correlates with the growth inhibitory effect of RA, suggesting that RAR β may be required for the growth inhibitory effect of retinoids in breast cancer cells.

How RAR β mediates the anti-proliferation effect

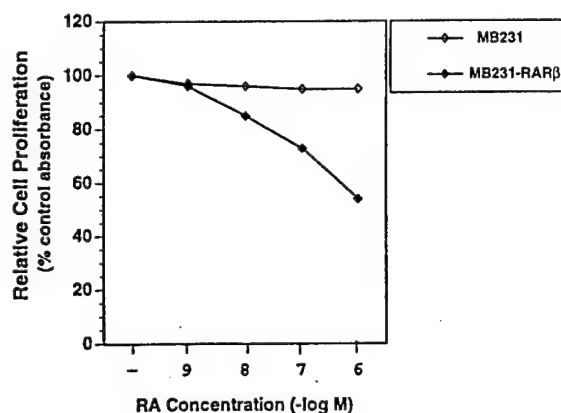


Fig. 3. Stable expression of RAR β gene confers RA response of hormone-independent breast cancer cells. The cDNA for the RAR β gene was cloned into pRc/CMV expression vector (Invitrogen, San Diego, CA) so that the expression of the RAR β gene is under the control of CMV promoter. The vector also contains a neomycin resistance gene that allows transfected cells grow in the presence of G418. The resulting pRc/CMV-RAR β recombinant construct was then stably transfected into MB231 cells using calcium phosphate precipitation method [52]. Stable transfectants that express RAR β gene were determined by Northern-blot analysis (not shown). The growth of one of the stable transfectants and parental MB231 cells in the absence or presence of RA was determined as described in Fig. 2.

of retinoids is unclear. It is known that RARs, in response to RA, can inhibit the effect of tumor promoter TPA and the transcriptional activity of proto-oncogenes cJun and cFos, which are commonly associated with cell proliferation [70,71]. Loss of RAR activities could remove such a negative control mechanism and therefore enhance the proliferative activity of cells. Due to auto-regulation of RAR β by RA, the expression of RAR β may play a critical role in increasing RA sensitivity. Therefore, the failure of RA to up-regulate RAR β expression suggests that lung cancer and breast cancer cells may have lost most, if not all, their RA sensitivities. The association between altered RAR transcriptional activities and carcinogenesis has been recently demonstrated in APL cells where an abnormal RAR α fusion transcript, with altered transcriptional activity, is produced by the characteristic chromosomal translocation [72–75]. It was also reported that an abnormal low expression of RAR β receptor may contribute to neoplastic progression of human oral squamous cell carcinoma cell lines [76] and the tumorigenicity of papillomavirus 18 transformed HeLa cells [77]. Since RAR β was shown to function as a tumor suppressor gene in epidermoid lung carcinogenesis [78], a low expression level of RAR β may be also involved in lung cancer and breast cancer development.

To obtain direct evidence that the lack of the RAR β gene expression contributes to retinoid resistance in cancer cells, we stably transfected the RAR β gene into MB231 cells in which the RAR β gene is not expressed (Fig. 1B). The stable transfectants that expressed the exogenous RAR β gene were subject to analysis for their response to the inhibitory effect of RA on anchorage-dependent cell growth. Fig. 3 shows the growth response to RA of one of the stable transfectants. The parental MB231 cells and the cells transfected with empty vector alone (data not shown) did not show any response to RA. However, the growth of the MB231 cells that express the RAR β gene was significantly inhibited by RA in a similar way that observed in hormone-dependent cells (Fig. 2). In addition, their anchorage-independent growth on soft agar was also inhibited by RA (data not shown). Furthermore, we found that RA could promote apoptosis in MB231 cells when RAR β was expressed (not shown). These data, therefore, pro-

vide a direct evidence that the expression of the RAR β gene can mediate the growth inhibitory effect of retinoids in breast cancer cells.

5. Multiple mechanisms are responsible for the loss of the RAR β gene expression

Understanding how the expression of the RAR β gene is abnormally regulated in cancer cells is fundamental to restoring functional RAR activities in cancer cells and may lead to early detection and valuable diagnostic markers of cancer cells. Although the mechanism is presently unknown, our results (Fig. 4) suggest that multiple mechanisms may exist (Fig. 5).

5.1. Mutations in retinoid receptors

Mutations within nuclear receptor genes have been found in many different diseases and are associated with their diminished ligand responsiveness. An well known example is v-erb A. V-erb A is the highly mutated form of c-erb A. It does not bind T3 but functions as repressor of several member of the steroid/thyroid hormone receptor family [79]. In patients with generalized resistance to thyroid hormone (GRTH syndrome), single amino acid mutations of TR results in the expression of receptors with either reduced or no T3 binding activity [80]. Similarly, a single amino acid change in the ligand binding domain of RAR α dramatically alters its ligand binding and transcriptional activity [81], and is responsible for the loss of RA sensitivity. Furthermore, abnormal RAR α fusion transcript is involved in the development of APL. Therefore, the loss of RA response in inducing RAR β gene expression observed in lung cancer cells raises the possibility that these cells may contain mutations in RAR or RXR genes. When we investigated β RARE activity by transient transfection using a reporter containing the β RARE linked with tk promoter (β RARE-tk-CAT, [59]) in both RA 'responsive' (cancer cells in which endogenous RAR β gene expression could be enhanced by RA) and 'nonresponsive' (cancer cells in which endogenous RAR β gene expression could not be enhanced by RA) lung cancer cell lines, we observed a strong reduced RA response in H292 cells (Fig. 4A). The slight RA response observed

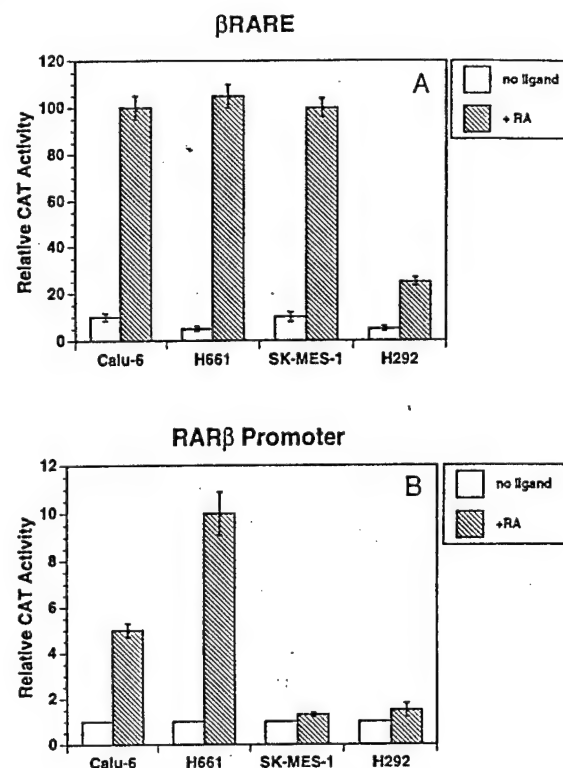


Fig. 4. Transcriptional activity of RARE and RAR β gene promoter in human lung cancer cell lines. β RARE-tk-CAT [59] (A) or *Bgl*III-*Bam*HI fragment of RAR β gene promoter [59] (B) linked with CAT gene were used as reporter genes to determine RA response in indicated human lung cancer cell lines. 2.0 μ g of β RARE-tk-CAT (A) or *Bgl*III-*Bam*HI-BS-CAT (B), 3.0 μ g of β -gal expression vector (pCH 110, Pharmacia) were transiently transfected into the indicated cells using the calcium phosphate precipitation method [52]. Cells were grown in the presence or absence of ligand (RA = 10^{-7} M). CAT activity was determined by a modified assay as described [52]. Counts per minute that were normalized for transfection efficiency by the corresponding β -gal activity were expressed as relative CAT activity. The data shown are means of three separate experiments.

may be due to amplification by the high copy number of the β RARE introduced by transient transfection and may not be biological significant. Since activation of the β RARE requires the binding of RAR/RXR heterodimers [52], and endogenous RARs and RXRs are highly expressed in H292 cells [66], the loss of the β RARE activity in H292 cells is likely due to mutations present in endogenous retinoid receptors. To study this possibility, we have examined the β RARE binding complexes formed by

nuclear proteins prepared from either H292 or Calu-6, H661, and SK-MES-1 cells. Our results (Lee, M-O, and Zhang, X-k. Unpublished data) demonstrate that H292 nuclear proteins formed a very different complex with β RARE as compared to other cell lines. Thus, H292 cells may contain mutated retinoid receptor(s) that may have lost their ligand responsiveness.

5.2. Alteration of retinoid receptor co-activators

The observation that β RARE-tk-CAT can be activated by RA in RA nonresponsive cancer cell SK-MES-1 (Fig. 4A) suggests that the cells contain necessary factors to activate RARE. Since activation of RARE requires the binding of RAR/RXR heterodimers [52] and RAR α , RAR γ and RXR genes were well expressed in SK-MES-1 cells [66], the presence of RAR α , RAR γ and RXRs in the cells may not be sufficient to render them responsive to RA. Although the underlying mechanism is unclear, similar observations have been made in other cell types. RAR α was found to be expressed in most human leukemia cells regardless of their responsiveness to RA [82–84]. In melanomas, it was found that levels of constitutive expression of RAR α and RAR γ were similar among RA resistant and RA-sensitive

cells [85]. These observations and the present finding that endogenous RARs and RXRs could not activate RAR β gene promoter suggests that other factors, other than RARs and RXRs, are required to mediate the transactivation function of RARs and RXRs in the RAR β gene promoter. These factors that may function as either activators or repressors on the RAR β promoter appear to be missing or mutated in SK-MES-1 cells. The nature of these factors is presently unknown. However, it has been suggested that retinoid receptors require co-activator(s) for efficient transactivation activity. These co-activators may function as 'bridge' to mediate the interaction between retinoid receptors and the basic transcriptional machinery in a promoter or response element specific manner and thereby allow the transactivation function of retinoid receptors on a particular target gene. E1A like protein has been reported to function as co-activator of RA-dependent RAR β promoter transactivation [86,87]. Although our data (not shown) suggest that E1A is not responsible for the altered RA response in inducing RAR β gene expression in lung cancer cells, our preliminary results (data not shown) show that there are several high molecular weight proteins that can interact with RAR in lung cancer cells. Interestingly, some of these high molecular weight proteins are not expressed in SK-

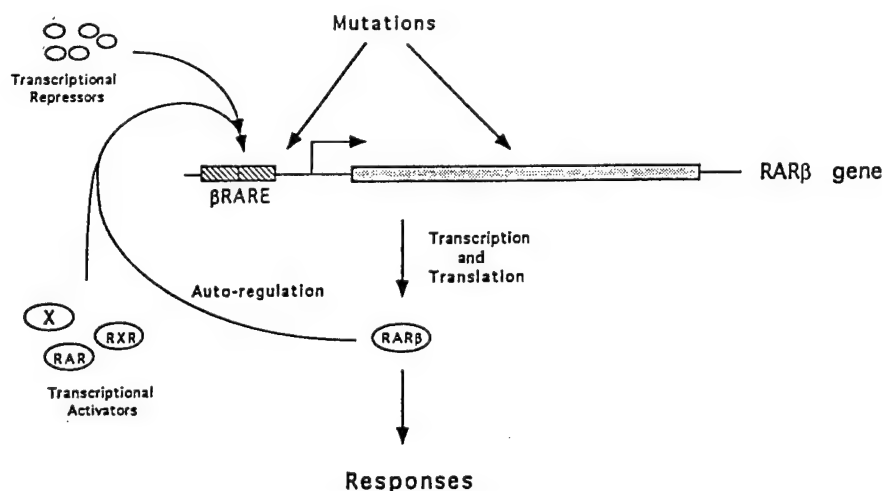


Fig. 5. Possible alterations in the regulation of RAR β gene expression in lung cancer cells. The expression of the RAR β gene in response to RA is mainly regulated by a RARE (β RARE) present in its promoter. The RAR β gene product, other retinoid receptors (RAR and RXR) and their co-activators (X) are involved in positive regulation of RAR β gene expression, resulting in amplification of RA responses. Lack of transcriptional activators (RAR, RXR and X) or mutations present in these activators and in RAR β gene promoter as well as overexpression of transcriptional repressors may lead to abnormal expression of the RAR β gene.

MES-1 cells. Thus, it is expected that these unknown RAR interacting proteins which may function as retinoid receptor co-activators on the β RARE likely play an important role in mediating RA response, and that lack or alteration of these proteins as well as overexpression of an unknown transcriptional repressor on the RAR β promoter may be responsible for the impaired RA response in inducing RAR β gene expression in some lung cancer cell lines.

5.3. Mutations in the RAR β gene promoter

The loss of RAR β gene expression in certain lung cancer cell lines may be due to mutations in the RAR β gene promoter so that the RAR β gene can not be activated. When a reporter containing the RAR β gene promoter (-745 to 156) (Fig. 4B, [59]) linked with CAT gene was transiently transfected in both RA responsive (Calu-6) and nonresponsive (H661, SK-MES-1 and H292) cancer cell lines, a strong induction of reporter CAT activity was observed in RA nonresponsive H661 cells while treatment of RA did not induce any CAT activity in other RA nonresponsive cell lines SK-MES-1 and H292 (Fig. 4B). No detectable expression of RAR β was observed in H661 cells either in the absence or presence of RA (Fig. 1A) while cotransfected β RARE-tk-CAT or RAR β promoter reporter was activated in response to RA. These observations suggest that H661 cells contain all necessary factors to activate β RARE and RAR β gene promoter (-745 to +156). Thus, the loss of RA response observed in this cell line may be due to a very different mechanism, most likely a mutation(s) in the endogenous RAR β promoter, from that of H292 or SK-MES-1 cells. However, contribution of negative regulatory elements present in the other region of the RAR β gene promoter or in the coding region can not be excluded.

6. Conclusion

Retinoids have shown promise as chemotherapeutic agents for a variety of human malignancies. However, no significant activity has been observed when they are used in the treatment of patients with advanced cancer. Our results demonstrate that lack

of RAR β gene expression and regulation by RA is a common feature associated with both lung cancer and breast cancer cells and may be one of the major factors responsible for retinoid resistance in these cells. Our results also demonstrate that RAR β can mediate the growth inhibitory effect of RA and exert anti-tumorigenic activities in breast cancer cells. Thus, the expression level of RAR β and its response to RA regulation may be used to determine the degree of RA sensitivities in cancer cells, and a lack of RAR β may contribute to cancer development. Our observations also provide valuable directions for developing retinoids in the treatment of human lung cancer and breast cancer. Further analysis of the RA response of various cancer cells introduced with RAR β will significantly enhance our understanding of the relationship between RAR β and RA sensitivity of cancer cells. Furthermore, a better understanding of the mechanism by which the expression of RAR β gene is regulated will help restoring functional RAR β activities in cancer cells.

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References

- [1] Lotan, R. Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* 605: 33-91, 1981.
- [2] Roberts, A.B. and Sporn, M.B. Cellular biology and biochemistry of the retinoids. In: M.B. Sporn, A.B. Roberts and D.S. Goodman (Eds.), *The Retinoids*, Vol. 2, pp. 209-286, Academic Press, Florida, 1984.
- [3] Lippman, S.M., Kessler, J.F. and Meyskens, F.L. Retinoids as preventive and therapeutic anticancer agents. *Cancer Treat. Rep.* 71: 493-405 (Part 1); 493-515 (Part 2), 1987.
- [4] Huang, M., Ye, Y.C., Chen, S.R., Chai, J.R., Lu, J.X., Zhao, L., Gu, L.J. and Wang, Z.Y. Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72: 567-572, 1988.
- [5] Castaigne, S., Chomienne, C., Daniel, M.T., Ballerini, P., Berger, R., Fenaux, P. and Degos, L. All-*trans* retinoic acid

- as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood* 76: 1704-1709, 1990.
- [6] Chomienne, C., Ballerini, P., Balitrand, N., Amar, M., Bernard, J.F., Boivin, P., Daniel, M.T., Berger, R., Castaignes and Degos, L. Retinoic acid therapy for promyelocytic leukemia. *Lancet* ii: 746, 1989.
 - [7] Warrell, R.P. Jr., Frankel, S.R., Miller, W.H., Jr., Scheinberg, D.A., Itri, L.M., Hittelman, W.N., Vyas, R., Andereff, M., Tafuri, A., Jakubowski, A., Gabrilove, J., Gordon, M., Dmitrovsky, E. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans* retinoic acid). *N. Engl. J. Med.* 324: 1385, 1991.
 - [8] Lippman, S., Kavanagh, J., Paredes-Espinoza, M. et al. 13-*cis*-retinoic acid plus interferon- α -2a: highly active systematic therapy for squamous cell carcinoma of the cervix. *J. Natl. Cancer Inst.* 84: 241-245, 1992.
 - [9] Lippman, S., Parkinson, D., Itri, L. et al. 13-*cis*-retinoic acid and interferon α -2a: effective combination therapy for advanced squamous cell carcinoma of the skin. *J. Natl. Cancer Inst.* 84: 235-241, 1992.
 - [10] Hong, W.K. et al. 13-*cis*-retinoic acid in the treatment of oral leukoplakia. *N. Engl. J. Med.* 315: 1501-1505, 1986.
 - [11] Hong, W.K., Lippman, S.M., Itri, L.M., Karp, D., Lee, J.S., Byers, R., Schantz, S.P., Kramer, A.M., Lotan, R., Peters, L.J., Dimery, I.W., Brown, W.B. and Goepfert, H. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.* 323: 795-801, 1990.
 - [12] Mettlin, C., Graham, S., Swanson, M. Vitamin A and lung cancer. *J. Natl. Cancer Inst.* 62: 1435-1438, 1979.
 - [13] Shekelle, R.B., Liu, S., Raynor, W.R. Jr., Lepper, M., Maliza, C., Rosof, A.H., Paul, O., Shyrock, A.M. and Stamler, J. Dietary vitamin A and risk of cancer in the Western Electric Study. *Lancet* ii: 1185-1189, 1981.
 - [14] Kard, J.D., Smith, A.H., Switzer, B.R. and Hanes, C.G. Serum vitamin A (retinol) and cancer incidence in Evans County, Georgia. *J. Natl. Cancer Inst.* 66: 7-16, 1981.
 - [15] Gerber, M., Cavallo, F., Marubini, E., Richardson, S., Barbieri, A., Capitelli, E., Costa, A., Crastes de Paulet, A., Crastes de Paulet, P., Decarli, A., Pastorino, U. and Pujol, H. Liposoluble vitamins and lipid parameters in breast cancer. A joint study in northern Italy and southern France. *Int. J. Cancer* 42, 489-494, 1988.
 - [16] Lansnizki, I. and Bollag, W. Prevention and reversal by a retinoid of 3,4-benzpyrene- and cigarette smoke condensate-induced hyperplasia and metaplasia of rodent respiratory epithelia in organ culture. *Cancer Treat. Rep.* 66: 1375-1380, 1982.
 - [17] Saffiotti, U., Montesano, R., Sellakumar, A.R. and Borg, S.A. Experimental cancer of the lung: inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous tumors. *Cancer (Phila.)* 20: 857-864, 1967.
 - [18] Moon, R.C. and Mehta, R.G. Chemoprevention of mammary cancer by retinoids. *Basic Life Sci.* 52, 213-224, 1990.
 - [19] Costa, A. Breast cancer chemoprevention. *Eur. J. Cancer* 29A: 589-592, 1993.
 - [20] Chopra, D.P. Squamous metaplasia in organ cultures of vitamin A-deficiency hamster trachea: cytokinetics and ultrastructural alteration. *J. Natl. Cancer Inst.* 69: 895-901, 1982.
 - [21] Gouveia, J., Hercend, T., Lemaigre, G., Mathe, G., Gros, F., Santelli, G., Homasson, J.P., Angebault, M., Lededente, A., Parrot, R., Gaillard, J.P., Bonniot, J.P., Marsac, J. and Pretet, S. Degree of bronchial metaplasia in heavy smokers and its regression after treatment with a retinoid. *Lancet* i: 710-712, 1982.
 - [22] Terasaki, T., Shimosato, Y., Nakajima, T., Tsumuraya, M., Ichinose, H., Nagatsu, T. and Kato, K. Reversible squamous cell characteristics induced by vitamin A deficiency in a small cell lung cancer cell line. *Cancer Res.* 47: 3533-3577, 1987.
 - [23] Doyle, L.A., Giangiulo, D., Hussain, A., Park, H.-J., Chiu Yen, R.-W. and Boeges, M. Differentiation of human variant small cell lung cancer cells lines to a classic morphology by retinoic acid. *Cancer Res.* 49: 6745-6751, 1989.
 - [24] Saccomanno, G., Moran, P.G., Schmidt, R.D. Effects of 13-*cis* retinoids on premalignant and malignant cells of lung origin. *Acta Cytol.* 26: 78-85, 1982.
 - [25] Marth, C., Bock, G. and Daxenbichler, G. Effect of 4-hydroxyphenylretinamide and retinoic acid on proliferation and cell cycle of cultured human breast cancer cells. *J. Natl. Cancer Inst.* 75: 871-875, 1985.
 - [26] Wetherall, N.T. and Taylor, C.M. The effects of retinoid treatment and antiestrogens on the growth of T47D human breast cancer cells. *Eur. J. Cancer Clin. Oncol.* 22: 53-59, 1986.
 - [27] Fontana, J.A. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation. *Exp. Cell. Biol.* 55: 136-144, 1987.
 - [28] Fontana, J.A., Hobbs, P.D. and Dawson, M.I. Inhibition of mammary carcinoma growth by retinoidal benzoic acid derivatives. *Exp. Cell. Biol.* 56: 254-263, 1988.
 - [29] Fontana, J.A., Mezu, A.B., Cooper, B.N. and Miranda, D. Retinoid modulation of estradiol-stimulated growth and of protein synthesis and secretion in human breast carcinoma cells. *Cancer Res.* 50: 1997-2002, 1990.
 - [30] Koga, M. and Sutherland, R.L. Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D3 or antiestrogen to inhibit human breast cancer cell proliferation. *J. Steroid Biochem. Mol. Biol.* 39: 455-460, 1991.
 - [31] Fontana, J.A. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation [1]. *Expl. Cell. Biol.* 55: 136-144, 1987.
 - [32] Fontana, J.A., Mezu, A.B., Cooper, B.N. and Miranda, D. Retinoid modulation of estradiol-stimulated growth and of protein synthesis and secretion in human breast carcinoma cells [1]. *Cancer Res.* 50: 1997-2002, 1990.
 - [33] Koga, M. and Sutherland, R.L. Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D3 or antioestrogen to inhibit T-47D human breast cancer cell proliferation. *J. Steroid Biochem. Mol. Biol.* 39: 455-460, 1991.
 - [34] Butler, W.B. and Fontana, J.A. Responses to retinoic acid of tamoxifen-sensitive and -resistant sublines of human breast cancer cell line MCF-7 [1]. *Cancer Res.* 52: 6164-6167, 1992.

- [35] Modiano, M., Dalton, W., Lippman, S. et al. Phase II study of fenretinide (*n*-[4-hydroxyphenyl]retinamide) in advanced breast cancer and melanoma. *Invest. New Drugs* 8: 317-319, 1990.
- [36] Cassidy, J., Lippman, M., LaCroix, A. et al. Phase II trial of 13-*cis*-retinoic acid in metastatic breast cancer. *Eur. J. Cancer Clin. Oncol.* 18: 925-928, 1982.
- [37] Goodman, G. Phase II trial of retinol in patients with advanced cancer. *Cancer Treat. Rep.* 70: 1023-1024, 1986.
- [38] Grunberg, S., Itri, L. Phase II study of isotretinoin in the treatment of advanced nonsmall cell lung cancer. *Cancer Treat. Rep.* 71: 1097-1098, 1987.
- [39] Petkovich, M., Brand, N.J., Krust, A. and Chambon, P. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330: 444-450, 1987.
- [40] Giguere, V., Ong, E.S., Seigi, P. and Evans, R.M. Identification of a receptor for the morphogen retinoic acid. *Nature* 330: 624-629, 1987.
- [41] Benbrook, D., Lernhardt, E. and Pfahl, M. A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature* 333: 624-629, 1988.
- [42] Brand, N., Petkovich, M., Krust, A., de The, H., Marchio, A., Tiollais, P. and Dejean, A. Identification of a second human retinoic acid receptor. *Nature* 332: 850-853, 1988.
- [43] Krust, A., Kastner, P.H., Petkovich, M., Zelent, A. and Chambon, P. A third human retinoic acid receptor, hRAR γ . *Proc. Natl. Acad. Sci. USA* 86: 5310-5314, 1989.
- [44] Giguere, V., Shago, M., Zirngibl, R., Tatz, J.P., Rossant, J. and Varmuza, S. Identification of a novel isoform of the retinoic acid receptor γ expressed in the mouse embryo. *Mol. Cell. Biol.* 10: 2335-2340, 1990.
- [45] Hamada, K., Gleason, S.L., Levi, B.Z., Hirschfeld, S., Apella, E. and Ozato, K. H-2RIIBP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. *Proc. Natl. Acad. Sci. USA* 86: 8289-8293, 1989.
- [46] Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345: 224-229, 1990.
- [47] Yu, V.C., Delsert, C., Andersen, B., Holloway, J.M., Devary, O.V., Naar, A.M., Kim, S.-Y., Boutin, J.-M., Glass, C.K. and Rosenfeld, M.G. RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 67: 1251-1266, 1991.
- [48] Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.M., Mader, S. and Chambon, P. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 68: 377-395, 1992.
- [49] Mangelsdorf, D.J., Borgmeyer, U., Heyman, R.A., Zhou, J.Y., Ong, E.S., Oro, A.E., Kakizuka, A. and Evans, R.M. Characterization of three RXR genes that mediate the action of 9-*cis*-retinoic acid. *Genes Dev.* 6: 329-344, 1992.
- [50] Evans, R.M. The steroid and thyroid hormone receptor family. *Science* 240: 889-895, 1988.
- [51] Green, S. and Chambon, P. Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet.* 4: 309-314, 1988.
- [52] Zhang, X.-k., Hoffmann, B., Tran, P., Graupner, G. and Pfahl, M. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* 355: 441-446, 1992.
- [53] Kliewer, S.A., Umesono, K., Mangelsdorf, D.L. and Evans, R.M. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signaling. *Nature* 355: 446-449, 1992.
- [54] Marks, M.S., Hallenbeck, P.L., Nagata, T., Segars, J.H., Apella, E., Nikodem, V.M. and Ozato, K. H-2RIIBP (RXR β) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J.* 11: 1419-1435, 1992.
- [55] Bugge, T.H., Pohl, J., Lonnoy, O. and Stunnenberg, H.G. RXR α , a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J.* 11: 1409-1418, 1992.
- [56] Zhang, X.-k. and Pfahl, M. Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.* 4: 156-162, 1993.
- [57] de The, H., Vivanco-Ruiz, M.M., Tiollais, P., Stunnenberg, H. and Dejean, A. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature* 343: 177-180, 1990.
- [58] Sucov, H.M., Murakami, K.K. and Evans, R.M. Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc. Natl. Acad. Sci. USA* 87: 5392-5396, 1990.
- [59] Hoffmann, B., Lehmann, J.M., Zhang, X.-k., Hermann, T., Graupner, G. and Pfahl, M. A retinoic acid receptor-specific element controls the retinoic acid receptor- β promoter. *Mol. Endocrinol.* 4: 1734-1743, 1990.
- [60] de The, Marchio, A., Tiollais, P. and Dejean, A. Differential expression and ligand regulation of the retinoic acid receptor α and β genes. *EMBO J.* 8: 429-433, 1989.
- [61] Haq, R.U., Pfahl, M. and Chytil, F. Retinoic acid affects the expression of nuclear retinoic acid receptors in tissues of retinol deficient rats. *Proc. Natl. Acad. Sci. USA* 88: 8272-8276, 1991.
- [62] Zhang, X.-k., Liu, Y., Lee, M.-O. and Pfahl, M. A specific defect in the retinoic acid response associated with human lung cancer cell lines. *Cancer Res.* 54: 5663-5669, 1994.
- [63] Nervi, C., Volleberg, T.M., George, M.D., Zelent, A., Chambon, P. and Jetten, A.M. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp. Cell Res.* 195: 163-170, 1991.
- [64] Houle, B., Leduc, F. and Bradley, W.E.C. Implication of RAR β in epidermoid (squamous) lung cancer. *Genes, Chr. Cancer* 3: 358-366, 1991.
- [65] Gebert, J.F., Moghal, N., Frangioni, J.V., Sugarbaker, D.J. and Neel, B.G. High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* 6: 1859-1868, 1991.
- [66] Geradts, J., Chen, J.Y., Russell, E.K., Yankaskas, J.R., Nieves, L. and Minna, J.D. Human lung cancer cell lines

- exhibit resistance to retinoic acid treatment. *Cell Growth Differ.* 4: 799-809, 1993.
- [67] Roman, S.D., Clarke, C.L., Hall, R.E., Alexander, I.E. and Sutherland, R.L. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.* 52: 2236-2242, 1992.
- [68] van der Burg, B., van der Leede, B.-j. M., Kwakkenbox-Isbrucker, L., Salverda, S., de Laat, S.W. and van der Saag, P.T. Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol. Cell. Endocrinol.* 91: 149-157, 1993.
- [69] Sheikh, M.S., Shao, Z.M., Chen, J.C., Hussain, A., Jetten, A.M. and Fontana, J.A. Estrogen receptor-negative breast cancer cells transfected with the estrogen receptor exhibit increased RAR α gene expression and sensitivity to growth inhibition by retinoic acid. *J. Cell Biochem.* 53: 393-404, 1993.
- [70] Yang-Yen, H.F., Zhang, X.-k., Graupner, G., Tzukerman, M., Sakamoto, B., Karin, M. and Pfahl, M. Antagonism between retinoic acid receptors and AP-1: Implications for tumor promotion and inflammation. *New Biol.* 3: 1206-1219, 1991.
- [71] Schule, R., Rangarajan, P., Yang, N., Klierer, S., Ransone, L.J., Bolado, J., Verma, I.M. and Evans, R.M. Retinoic acid is a negative regulator of AP-1 responsive genes. *Proc. Natl. Acad. Sci. USA* 88: 6092-6096, 1991.
- [72] Borrow, J., Goddard, A.D., Sheer, D. and Sonomon, E. Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. *Science* 249: 1577-1580, 1990.
- [73] Alcalay, M., Zangrilli, D., Pandolfi, P.P., Longo, L., Mencarelli, A., Giacomucci, A., Rocchi, M., Biondi, A., Rambaldi, A., Lo Coco, F., Diverio, D., Donti, E., Grignani, F. and Pelicci, P.G. Translocation breakpoint of acute promyelocytic leukemia lies within the retinoic acid receptor α locus. *Proc. Natl. Acad. Sci. USA* 88: 1977-1981, 1991.
- [74] de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. The PML-RAR α fusion mRNA generated by the t(15:17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* 66: 675-684, 1991.
- [75] Kakizuka, A., Miller, W.H., Jr., Umesono, K., Warrell, R.P., Jr., Frankel, S.R., Murty, V.V.V.S., Dmitrovsky, E. and Evans, R.M. Chromosomal translocation t(15:17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcriptional factor. PML. *Cell* 66: 663-674, 1991.
- [76] Hu, L., Crowe, D.L., Rheinwald, J.G., Chambon, P. and Gudas, L. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.* 51: 3972-3981, 1991.
- [77] Bartsch, D., Boye, B., Baust, C., Hausen, H. and Schwarz, E. Retinoic acid-mediated repression of human papillomavirus 18 transcription and different ligand regulation of the retinoic acid receptor β gene in non-tumorigenic and tumorigenic HeLa hybrid cells. *EMBO J.* 11: 2283-2291, 1992.
- [78] Houle, B., Rochette-Egly, C. and Bradley, W.E.C. Tumor-suppressive effect of the retinoic acid receptor β in human epidermoid lung cancer cells. *Proc. Natl. Acad. Sci. USA* 90: 985-989, 1993.
- [79] Damm, K., Thompson, C.C. and Evans, R.M. Protein encoded by v-erbA functions as a thyroid hormone antagonist. *Nature* 339: 593-597, 1989.
- [80] Sakurai, A., Takeda, K., Ain, K., Ceccarelli, P., Nakai, A., Seino, S., Bell, G.I., Refetoff, S. and DeGroot, L.J. Generalized resistance to thyroid hormone associated with a mutation in the ligand binding domain of the human thyroid hormone receptor β . *Proc. Natl. Acad. Sci. USA* 86: 8977-8981, 1989.
- [81] Pratt, M.A.C., Kralova, J. and McBurney, M.W. A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. *Mol. Cell. Biol.* 10: 6445-6453, 1990.
- [82] Gallagher, R.E., Said, F., Pua, I., Papenhausen, P.R., Paietta, E., Wiernik, P.H. Expression of retinoic acid receptor α -mRNA in human leukemia cells with variable responsiveness to retinoic acid. *Leukemia* 3: 789, 1990.
- [83] Largent, C., Detmer, K., Corral, J.C., Hack, F.M., Lawrence J. Expression of retinoic acid receptor alpha mRNA in human leukemia cells. *Blood* 74: 99, 1989.
- [84] Wang, C., Curtis, J.E., Minden, M.D., McCulloch, E.A. Expression of a retinoic acid receptor gene in myeloid leukemia cells. *Leukemia* 264: 1989.
- [85] Clifford, E.A., Petkovich, M., Chambon, P., Lotan, R. Modulation by retinoids of mRNA levels for nuclear retinoic acid receptors in murine melanoma cells. *Mol. Endocrinol.* 4: 1546, 1990.
- [86] Berkenstam, A. et al. Cooperativity in transcription between retinoic acid receptor and TFIID requires an activity analogous to E1A. *Cell* 69: 401-412, 1992.
- [87] Krut, F.A.E. et al. E1a functions as a coactivator of retinoic acid-dependent retinoic acid receptor-b2 promoter activation. *Mol. Endocrinol.* 7: 604-615, 1993.

Correlation of Retinoid Binding Affinity to Retinoic Acid Receptor α with Retinoid Inhibition of Growth of Estrogen Receptor-positive MCF-7 Mammary Carcinoma Cells¹

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ABSTRACT

Both anchorage-dependent growth and anchorage-independent growth of the estrogen receptor-positive mammary carcinoma cell line MCF-7 are inhibited by all-*trans*-retinoic acid. This cell line has nuclear retinoic acid receptors (RARs) α and γ . The natural retinoids all-*trans*-retinoic acid and 9-*cis*-retinoic acid and a series of 12 conformationally restricted retinoids, which showed a range of binding selectivities for these receptors and had either agonist or antagonist activity for gene transcriptional activation by the RARs, were evaluated for their abilities to inhibit anchorage-dependent (adherent) and anchorage-independent (clonal) growth of MCF-7 cells. Correlation analyses were performed to relate growth inhibition by these retinoids with their binding affinity to RAR α or RAR γ . Inhibition of anchorage-dependent growth in culture after 7 days of retinoid treatment correlated with binding to RAR α ($n = 14$; $P \leq 0.001$) and not to RAR γ ($n = 14$; $P > 0.1$). Both the RAR α -selective retinoid agonists and the two RAR antagonists that were evaluated inhibited adherent cell growth. The RAR γ -selective agonists had very low growth inhibitory activity ($< 10\%$) at concentrations as high as 12.5 μM . These results suggest that RAR α is the retinoid receptor involved in the inhibition of adherent cell growth by retinoids and that transcriptional activation by this receptor on a RAR response element does not appear to be required for this process to occur. For this series of retinoids, inhibition of anchorage-independent growth after 21 days of retinoid treatment only correlated ($n = 12$; $P \leq 0.005$) with binding affinity to RAR α for the retinoid agonists, although the RAR γ -selective retinoids displayed weak activity. The RAR antagonists were very poor inhibitors of growth. These results suggest that activation of gene transcription by RAR α appears to be required for inhibition of anchorage-independent growth by retinoids in this estrogen receptor-positive mammary carcinoma cell line.

INTRODUCTION

trans-RA³ inhibits the growth of ER⁺ breast cancer cells. The inhibition of cell proliferation by *trans*-RA and other retinoids was not

due to general cytotoxicity because cell division continued (1); for example, the doubling time of MCF-7 mammary carcinoma cells was increased 2-fold by 1 μM *trans*-RA (2). In addition, the cells reinitiated their normal growth rate upon removal of *trans*-RA (3). Although cell growth was retarded by *trans*-RA, the cells did not undergo typical differentiation to their more mature phenotype, as characterized by the enhanced expression of an epithelial membrane antigen (2).

In contrast, the estrogen E₂ stimulates ER⁺ breast cancer cell growth and proliferation. Transfection of an estrogen receptor construct into ER⁻ MDA-MB-231 breast cancer cells, which lack ERs and are not stimulated by E₂, permitted the inhibition of cell growth by *trans*-RA (4).

Retinoids exert their modulatory effects on cell growth by binding to the retinoid receptor nuclear proteins, of which there are two classes, the RARs and the RXRs, each of which has three subtypes (α , β , and γ). The RARs form heterodimers with the RXRs, which enhance their gene transcriptional responses (5, 6). The retinoid receptors may influence gene transcriptional activation by binding to specific DNA sequences (retinoid response elements, *i.e.*, RAREs and RXREs) and by affecting the activity of the transcription factor AP-1 (Jun/Fos) proteins, which bind to other DNA sites (reviewed in Ref. 7). Binding of both the natural retinoids *trans*-RA and 9-*cis*-RA and their synthetic analogues to the retinoid receptors enhances receptor responses (8–10).

ER⁻ breast cancer cells have high levels of RAR α mRNA, which are appreciably above those found in ER⁻ breast cancer cells. The levels of RAR γ mRNA in ER⁺ and ER⁻ cells vary, whereas those of RAR β are undetectable or extremely low in ER⁺ cells and variable in ER⁻ cells (4, 11). RA did not regulate RAR α and RAR γ mRNA levels in either ER⁺ or ER⁻ breast cancer cell lines but did induce a very low level of RAR β mRNA in ER⁺ T47D cells (4). In contrast, E₂ increased RAR α mRNA in T47D (12) and MCF-7 cell lines (11). Transfection of ER into ER⁻ MDA-MB-231 cells was accompanied by a 3-fold increase in RAR α mRNA, which then rose 12-fold on treatment with E₂ (4). Most important, RAR α mRNA levels were found by Shao *et al.* (12) and Roman *et al.* (13) to correlate positively with the ER⁺ status in a series of human breast cancer biopsy specimens. The MCF-7 cell line used in these studies has similar levels of RAR α and RAR γ mRNA (14, 15).

The ability of the natural retinoids to isomerize in cell culture (16) complicates the interpretation of results in experiments using *trans*-RA or 9-*cis*-RA. This problem can be avoided by using conformationally restricted retinoids, in which the bonds corresponding to the double bonds of the natural retinoids are constrained to particular configurations by inclusion in aromatic rings. Because the RARs do

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³ The abbreviations used are: *trans*-RA, all-*trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; ER⁺, ER⁻, estrogen receptor-positive and -negative, respectively; E₂, 17 β -estradiol; RAR, retinoic acid receptor; RARE, retinoic acid response element; NMR, nuclear magnetic resonance; FBS, fetal bovine serum; SR3983, (E)-4-[2-methyl-4-(2,6,6-trimethylcyclohexenyl)-1,3-butadienyl]benzoic acid; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid; Ro41-5253, (E)-6-[1-(4-carboxyphenyl)propen-2-yl]3,4-dihydro-4,4-dimethyl-7-heptyloxy-2H-1-benzothiopyran 1,1-dioxide; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid; [H₃]TTAB, [1',3',4'-H₃]4-(5',6',7',8'-tetrahydro-5',5',8',8'-tetramethyl-2'-anthracenyl)benzoic acid; TTNN, 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-naphthalenecarboxylic acid; Am580, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenecarboxamido)benzoic acid; Am80, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenamincarbonyl)benzoic acid; SR3986, (E)-4-[2-(4-(3-methylbutylthio)phenyl)propenyl]benzoic acid; SR11273, N-(5,6,7,8-tetrahydro-5,5,8,8-tetra-methyl-2-naphthalenyl)-N'-(4-carboxyphenyl)-diazene N-oxide; SR11274, N-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-N'-(4-

carboxyphenyl)diazene N'-oxide; SR11254, 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)(hydroxyimino)methyl]-2-naphthalenecarboxylic acid; SR11262, (E)-3-[4-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)phenyl]-2-propenoic acid.

not appear to substitute for each other in breast cancer cell lines (17), the use of retinoids that show selective binding affinity and/or transcriptional activation activity for particular retinoid receptors permits probing of the roles of the retinoid receptors in specific cell lines. This technique permitted Rudd *et al.* (18) to determine that of the two RARs (RAR α and RAR γ) present in the mouse epidermal cell line JB6, only gene transcriptional activation by the latter receptor in the presence of retinoids showed a statistically significant correlation with retinoid inhibition of clonal proliferation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate under anchorage-independent growth conditions. Similarly, retinoid receptor-selective retinoids should provide valuable information concerning the regulatory roles of retinoids in breast cancer cell proliferation. Here, we now show that the inhibition of both the anchorage-dependent (adherent) growth and the anchorage-independent (clonal) growth of MCF-7 mammary carcinoma cells correlate with the ability of retinoids to interact with RAR α , but evidently by different mechanisms.

MATERIALS AND METHODS

Retinoids. *trans*-RA (1) was obtained from Sigma Chemical Co. (St. Louis, MO) and recrystallized. 9-*cis*-RA (2) was synthesized as described (19). SR3983 (TCBB; 3) was prepared according to Dawson *et al.* (20). TTNPB (Ro13-7410; 4) (21) and Ro41-5253 (11) (22) were generously provided by Hoffmann-La Roche, Nutley, NJ. TTAB (5) and TTNN (SR3957; 6) were synthesized as reported (23, 24). Am580 (7) and Am80 (8) were synthesized by the procedures of Kagechika *et al.* (25). SR3986 (12) (26) and [^3H]TTAB (27) were prepared as described.

SR11273 (9) and SR11274 (10) were synthesized (28) as a 1:1 mixture, which was separated by reversed-phase HPLC (Waters Radialpak Novapak C₁₈, 8 x 100-mm column, 0.1% CH₃CO₂H/90% methanol, 1 ml/min, 260 nm) to give 9 as pale-yellow needles: melting point, 248–250°C; IR (KBr) 3436, 2930, 1686 cm⁻¹; ^1H NMR (300 MHz, CDCl₃) δ 1.33 [s, 6, C(CH₃)₂], 1.36 [s, 6, C(CH₃)₂], 1.74 (s, 4, CH₂CH₂), 7.44 (d, J = 8.8 Hz, 1, ArH), 8.04 (dd, J = 2.5, 8.8 Hz, 1, ArH), 8.11 (d, J = 8.8 Hz, 2, ArH), 8.22 (d, J = 8.8 Hz, 2, ArH), 8.24 (d, J = 2.5 Hz, 1, ArH); and 10 as yellow needles: melting point, 240–242°C; IR (KBr) 3437, 2959, 1688 cm⁻¹; ^1H NMR (300 MHz, CDCl₃) δ 1.33 [s, 6, C(CH₃)₂], 1.35 [s, 6, C(CH₃)₂], 1.73 (s, 4, CH₂CH₂), 7.43 (d, J = 9.2 Hz, 1, ArH), 8.14 (m, 2, ArH), 8.26 (d, J = 8.8 Hz, 2, ArH), 8.42 (d, J = 8.8 Hz, 2, ArH).

SR11254 (13) was synthesized by treatment of methyl 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylate with HONH₂·HCl and NaOAc in methanol (24 h, 60°C) to give the two isomeric oxime esters (93%) after chromatography (CH₂Cl₂), followed by hydrolysis of the esters (aq. KOH in ethanol, 0.5 h, 70°C) and acidification (1 N HCl) to give the two oxime carboxylic acids (95% as a 1.2:1 mixture) after crystallization (CH₂Cl₂/hexane): melting point, 238–240°C; IR (KBr) 3422, 2961, 1890 cm⁻¹; ^1H NMR (300 MHz, acetone-*d*₆) δ 1.24 [s, 6, C(CH₃)₂], 1.32 [s, 6, C(CH₃)₂], 1.75 [d, 4, (CH₂)₂], 7.25 (dd, J = 1.9, 8.2 Hz, 1, ArH), 7.37 (d, J = 8.2 Hz, 1, ArH), 7.58 (d, J = 1.9 Hz, 1, ArH), 7.65 (dd, J = 1.6, 8.5 Hz, 1, NaphH), 8.01 (br s, 1, NaphH), 8.12 (d, J = 8.5 Hz, 1, NaphH), 8.17 (dd, J = 1.6, 8.5 Hz, 1, NaphH), 8.23 (d, J = 8.5 Hz, 1, NaphH), 8.77 (br s, 1, NaphH) for major isomer and 1.32 [s, 6, C(CH₃)₂], 1.40 [s, 6, C(CH₃)₂], 1.81 [s, 4, (CH₂)₂], 7.29 (dd, J = 1.9, 8.2 Hz, 1, ArH), 7.46 (d, J = 1.9 Hz, 1, ArH), 7.53 (d, J = 8.2 Hz, 1, ArH), 7.88 (br s, 1, NaphH), 7.95 (d, J = 8.5 Hz, 1, NaphH), 7.99 (dd, J = 1.6, 8.5 Hz, 1, NaphH), 8.10 (dd, J = 1.6, 8.5 Hz, 1, NaphH), 8.12 (d, J = 8.5 Hz, 1, NaphH), 8.70 (br s, 1, NaphH) for minor isomer. Anal. (C₂₆H₂₇NO₃) C, H. The RAR γ -selectivity of this compound has also been reported by Ostrowski *et al.* (29).

SR11262 (14) (30, 31) was prepared by a Pd(0)-catalyzed coupling of 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthaleneboronic acid with ethyl (*E*)-3-(4-bromophenyl)propionate, using Pd[P(C₆H₅)₃]₄ and aq. Na₂CO₃ in refluxing MeO(CH₂)₂OMe for 12 h, followed by chromatography (5% EtOAc/hexane) to give the ethyl ester of SR11262 (93%), which was hydrolyzed (aq. KOH in methanol, 1 h, 80°C) and acidified (1 N HCl) to give SR11262 (93%) after crystallization (CH₂Cl₂/hexane): melting point, 262–265°C; IR (KBr)

2956, 1681, 1623 cm⁻¹; ^1H NMR (300 MHz, CDCl₃) δ 1.30 [s, 6, C(CH₃)₂], 1.34 [s, 6, C(CH₃)₂], 1.72 [s, 4, (CH₂)₂], 2.27 (s, 3, Me), 6.50 (d, J = 16.0 Hz, 1, C=CHCO₂), 7.17 (s, 1, ArH), 7.21 (s, 1, ArH), 7.41 (d, J = 8.2 Hz, 2, ArH), 7.61 (d, J = 8.2 Hz, 2, ArH), 7.85 (d, J = 16.0 Hz, 1, HC=CCO₂); HMRS for C₂₄H₂₈O₂ (MH⁺): calculated, 349.2167; found, 349.2169.

HPLC indicated that the peak area for each retinoid was greater than 99% of the total peak area. The retinoids were characterized by IR and ^1H NMR spectral and elemental analyses before dissolving in DMSO or ethanol/DMSO. Experiments using olefinic retinoids were conducted under yellow or subdued lighting.

Reagents. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Eagle's MEM, trypsin (Sigma Chemical Co., St. Louis, MO), FCS (GIBCO Laboratories, Grand Island, NY), FBS (Gemini Bio-Products, Calabasas, CA), DMEM (BioWhittaker, Walkersville, MD), and purified agar (Baltimore Biological Laboratories, Baltimore, MD) were purchased.

Cells. MCF-7 breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD).

Receptors. Human RAR α 1 was expressed in recombinant baculovirus-infected Sf9 *Spodoptera frugiperda* insect cells (32). cDNAs for recombinant human RAR β and human RAR γ were cloned in frame into the expression vector pGEX-2T (Pharmacia, Piscataway, NJ). *Escherichia coli* (DH5 α) transformed with pGEX-2T-RAR β or pGEX-2T-RAR γ and diluted 1:10 in LB medium containing ampicillin (100 $\mu\text{g}/\text{ml}$) was incubated at 37°C; after 1 h, isopropyl-1-thio- β -D-galactopyranoside was added (final concentration, 0.1 mM). At 5 h, bacterial cultures were pelleted by centrifugation and resuspended in 0.1 volume 20 mM Tris (pH 8.0), 100 mM NaCl, and 0.5% NP40. The bacteria were lysed and centrifuged. The fusion proteins were purified by adsorption on glutathione-agarose beads, which were then washed with ice-cold PBS, before elution with 50 mM Tris (pH 8.0)-5 mM reduced glutathione. Eluted protein was stored at -80°C.

Receptor Binding. Each RAR preparation (1–2 μg of total protein for RAR α , 4–6 μg for RAR β and RAR γ) was incubated (4°C, 6 h) with 2 nM [^3H]TTAB (27) (specific activity, 64 Ci/mmol) with or without unlabeled retinoid as described (33), except 0.1% gelatin replaced the ovalbumin. The bound [^3H]TTAB was separated from free [^3H]TTAB by gel filtration as described by Levin *et al.* (10).

Inhibition of Anchorage-dependent Growth by Retinoids. Cell suspensions (0.5–1.0 $\times 10^5$) in 4.0 ml MEM-5% FCS were incubated at 37°C in humidified 5% CO₂/95% air. After 24 h, the retinoids in 10 μl ethanol:DMSO (1:1) were added to each 60-mm dish. Retinoid concentrations ranged from 0.1–12.5 μM , and the control contained only vehicle. Media and retinoid solutions were replaced every third day. On day 8, cells were trypsinized and counted (3). Experiments were performed in triplicate, and concentrations required for 50% inhibition of cell growth (IC₅₀s) were determined by extrapolation or interpolation of dose curves of the means.

Inhibition of Anchorage-independent Colony Formation by Retinoids. Cells (1.0 $\times 10^4$) in 1.5 ml of DMEM, 0.3% agar, 10% FBS over 2.5 ml of DMEM, 0.5% agar, and 10% FBS were exposed to retinoids in 6.0 ml DMEM, 0.3% agar, and 10% FBS. Retinoids were dissolved in DMSO and diluted with medium. Retinoid concentrations ranged from 0.1–1000 nM, and $\leq 0.2\%$ DMSO was in these samples and in the control. Cultures were incubated at 37°C in 5% CO₂/95% air for 3 weeks. The unstable retinoids 1 through 3 were replaced every third day. On day 21, each culture was treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1.25 mg) in 1 ml PBS for 3–4 h, and the blue-stained colonies were counted. The number of colonies per dish was calculated by multiplying the average number per cm² by the 28-cm² dish area. Experiments were performed in triplicate.

RESULTS

In addition to *trans*-RA (1) and 9-*cis*-RA (2), 12 conformationally restricted retinoids were evaluated. Their structures are shown in Fig. 1. Retinoids 3 through 5 and 7 through 12 have a benzoic acid terminus in place of the 11E,13E-dienoic acid system of *trans*-RA to constrain this bond system to a *s*-cisoid conformation. The naphthalenecarboxylic acid rings of TTNN (6) and SR11254 (13) restrict the naphthalene bonds corresponding to the 9E,11E,13E-double bonds of *trans*-RA in cisoid conformations. The bonds of SR11262 (14) that

| Retinoid | RAR binding affinity | | | | MCF-7 cell growth inhibition | | | |
|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------------|-----------------------|-----------------------|-----------------------|
| | RAR α | | RAR γ | | Anchorage-dependent | | Anchorage-independent | |
| | Relative affinity (%) | IC ₅₀ (nM) | Relative affinity (%) | IC ₅₀ (nM) | 1 μ M retinoid (%) | IC ₅₀ (nM) | 1 nM retinoid (%) | IC ₅₀ (nM) |
| 1 (RA) | 67 | 2.8 | 89 | 0.6 | 54 | 1.2 | 88 | 0.08 |
| 2 (9- <i>cis</i> -RA) | 40 | 19 | 49 | 11 | 46 | 1.8 | 65 | 0.2 |
| 3 (SR3983) | 30 | 44 | 0 | 100 | 14 | 122 | 25 | 4.2 |
| 4 (TTNPB) | 56 | 7.0 | 56 | 8.2 | 54 | 0.77 | 91 | 0.4 |
| 5 (TTAB) | 63 | 2.9 | 97 | 0.78 | 53 | 2.1 | 98 | 0.04 |
| 6 (TTNN) | 4 | 115 | 35 | 24 | 15 | 6.0 | 16 | 10.2 |
| 7 (Am580) | 36 | 2.3 | 0 | 390 | 45 | 1.5 | 85 | 0.06 |
| 8 (Am80) | 29 | 88 | 3 | >1000 (3%) | 45 | 1.2 | 40 | 2.0 |
| 9 (SR11273) | 55 | 8.2 | 70 | 5.1 | 58 | 1.2 | 91 | 0.05 |
| 10 (SR11274) | 55 | 7.2 | 58 | 7.0 | 47 | 0.4 | 37 | 1.8 |
| 11 (Ro41-5253) | 54 | 7.8 | 0 | >1000 (3%) | 43 | 1.8 | 10 | >1000 (22%) |
| 12 (SR3986) | 14 | 100 | 38 | 21 | 7 | 15 | 8 | >1000 (18%) |
| 13 (SR11254) | 0 | >1000 (18%) | 52 | 20 | 2 | >12.5 (6%) | 27 | 65 |
| 14 (SR11262) | 0 | >1000 (38%) | 8 | 84 | 9 | >12.5 (9%) | 36 | 5.7 |

Fig. 1. Binding affinity to human RAR α and human RAR γ for 14 retinoids and their inhibition of MCF-7 mammary carcinoma cell growth. Relative binding affinity was determined as the percentage of [$1',3',4'$ - 3 H]TTAB displaced from RAR by 10 nM retinoid. IC₅₀s for retinoid binding to the RARs are retinoid concentrations required to inhibit binding of [$1',3',4'$ - 3 H]TTAB by 50%. IC₅₀s for cell growth inhibition by retinoids are the retinoid concentrations required to obtain 50% inhibition after retinoid treatment for 7 days for anchorage-dependent growth or for 21 days for anchorage-independent growth. Numbers in parentheses refer to the percentage of inhibition when the IC₅₀ was not reached.

correspond to the 9E,11E-double bonds of *trans*-RA also have s-cisoid geometry by being in a phenyl ring. In the tetrahydroanthracenyl analogue TTAB (5), the bonds corresponding to the 7E,9E-double bonds of *trans*-RA are s-transoid. Retinoids 4 through 10, 13, and 14 have s-cisoid bond geometry in their tetrahydronaphthalene bonds corresponding to the 5,7E-double bonds of *trans*-RA, whereas 11 and 12 have benzodihydrothiopyran and phenyl rings, respectively, that provide similar geometry. The two methyl groups at the tetrahydronaphthalene ring position corresponding to the 4-position of RA prevent deactivation at this position by metabolic oxidation. With the

exception of the retinoid antagonists 11 (22) and 12 (34), these retinoids have demonstrated retinoid activity in cell culture assays. Retinoids 1 through 6 (18), 13, and 14 inhibited the anchorage-independent growth of mouse epidermal JB6 cells induced by 12-*O*-tetradecanoylphorbol-13-acetate. The IC₅₀s for 13 and 14 were 42 and 30 nM, respectively (data not shown). Retinoids 7 through 10 induced the differentiation of HL-60 leukemia cells (25, 26). Antagonist 11 was inactive in the HL-60 assay (22). At 10^{-8} M, 12 showed no activity in the hamster tracheal organ culture reversal of keratinization assay, in which the IC₅₀ for TTNPB was 2×10^{-12} M (30), and did not activate the RARs for gene transcription in the cotransfection assay (34).

The abilities of these retinoids to inhibit the growth of MCF-7 cells were evaluated. Inhibition of anchorage-dependent growth was measured at the end of 7 days of retinoid treatment to distinguish more readily the relative inhibitory effects of the various receptor-selective retinoids. Concentrations in the micromolar range were required for the active retinoids to inhibit cell growth. Representative curves for MCF-7 cell growth inhibition in the presence of 1.25 μ M RAR α -selective retinoid Am580 (7) and RAR γ -selective SR11254 (13) are shown in Fig. 2. The RAR α -selective retinoid was more potent, with cell growth inhibition occurring earlier in the treatment period and at lower doses. There were also differences in the behavior of the cells to the retinoids. Cells treated with Am580 retained their ability to adhere to the culture plate although their growth was inhibited, whereas the SR11254- and SR11262-treated cells detached from the plate at the time that growth inhibition was observed. Growth inhibition by 1.25 μ M Am580 was 58% after 7 days and 76% after 10 days, while that by 1.25 μ M SR11254 was 5 and 25%, respectively, after these time periods. Inhibition by 12.5 μ M RAR γ -selective retinoid SR11254 (13) was 6% after 7 days and 34% after 10 days, and inhibition by 12.5 μ M RAR β , γ -selective SR11262 (14) was 9% after 7 days and 67% after 9 days. In Fig. 1 are given the concentrations of these 14 retinoids required to inhibit the anchorage-dependent growth of MCF-7 cells by 50% (IC₅₀s) and their percent cell growth inhibition at 1 μ M. Because general cytotoxicity, including detachment from the cell culture plates, was observed for several of these retinoids above 12.5 μ M concentrations, IC₅₀s were not determined for 13 and 14.

The abilities of these retinoids to inhibit the anchorage-independent growth of MCF-7 cells after 21 days of treatment were also assessed.

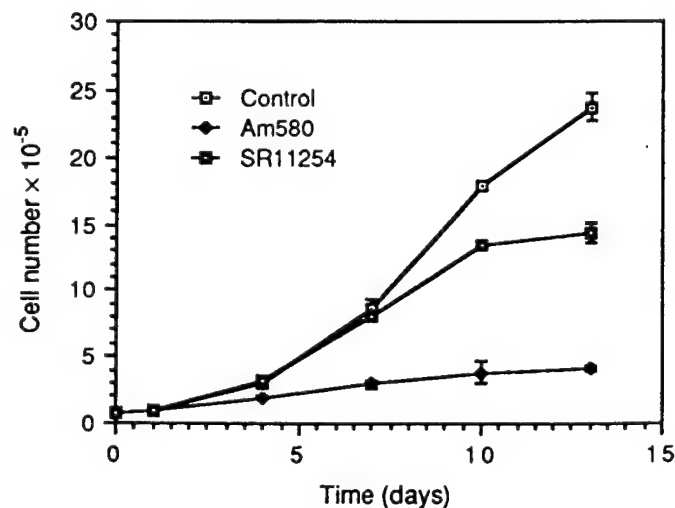


Fig. 2. Effects of 1.25 μ M Am580 (7) and SR11254 (14) on anchorage-dependent growth of MCF-7 mammary carcinoma cells in culture with time, compared to the nontreated control. Retinoids were replaced every 3 days.

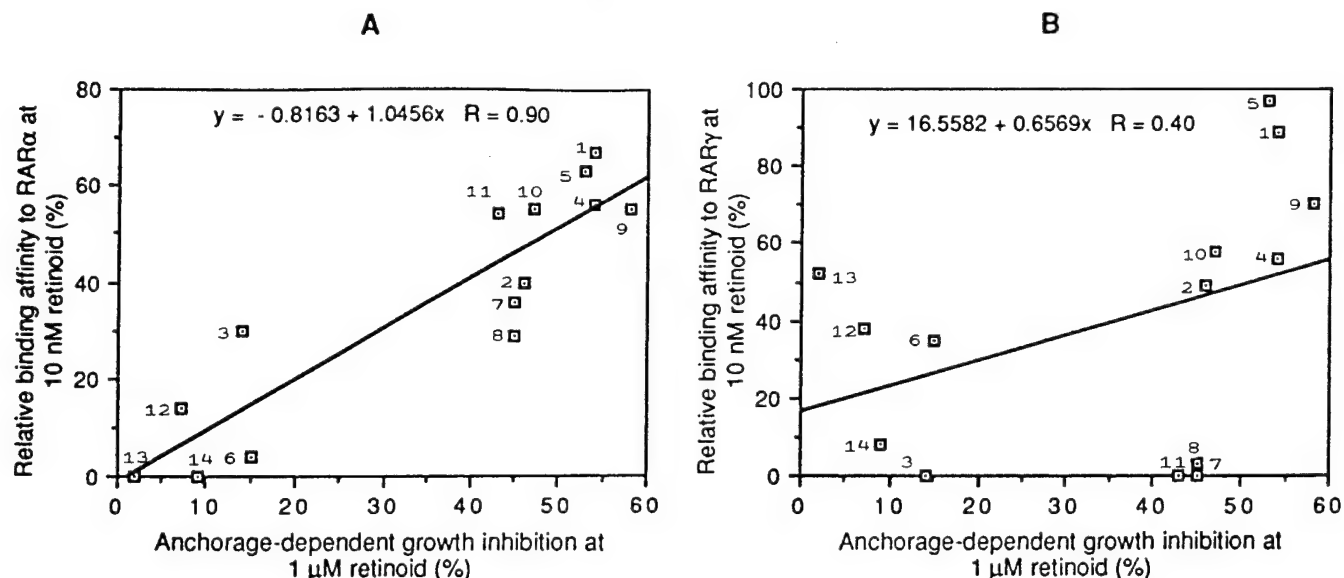


Fig. 3. Binding of retinoid agonists and antagonists to RAR α and RAR γ compared to their inhibition of MCF-7 mammary carcinoma cell anchorage-dependent growth. The relative RAR binding affinities of 14 retinoids, as measured by their ability to displace [^3H]TTAB bound to recombinant human RAR α (A) or human RAR γ (B), were correlated with their inhibition of anchorage-dependent cell growth at 1 μM , as measured by cell counting after 7 days of retinoid treatment.

The IC_{50} s for these compounds and their percentage of colony formation inhibition at 1 nM are also presented in Fig. 1. MCF-7 cells were far more sensitive to retinoids under anchorage-independent growth conditions (where IC_{50} s below 1 nM were observed for the most effective compounds) than under anchorage-dependent growth conditions.

The binding affinity of these retinoids for the recombinant human RARs was determined by measuring their abilities (at 10 nM retinoid concentrations) to displace competitively bound tritiated TTAB and to displace 50% of bound label (IC_{50} ; Fig. 1). *trans*-RA (1), 9-*cis*-RA (2), SR3983 (3), TTNPB (4), and TTAB (5) displayed similar binding affinity for both RAR α and RAR γ . RAR α selectivity was conferred to the two analogues Am580 (7) and Am80 (8) of TTNPB by replacement of the propenyl group linking the two aromatic ring systems of TTNPB by carboxamide and carbamoyl groups, respectively. The affinity of Am580 to both RAR α and RAR γ was at least 4-fold higher than that of Am80. Their more polar isosteres SR11273 (9) and SR11274 (10), having diazene *N*-oxide bonds in place of the amide bonds, had higher affinity for RAR α than either amide did but also had high affinity for RAR γ . The RAR α -selective antagonist Ro41-5253 (11) has a lipophilic pendant heptyloxy group and a sulfone function at its ring position corresponding to the 4-position of RA that apparently permit binding to but not activation of RAR α . The antagonist SR3986 (12) has a lipophilic 3-methylbutyl group at its lipophilic terminus that permits binding to both RAR α and RAR γ but not receptor activation (34). SR3986 bound to RAR γ 4-fold more avidly than to RAR α . The presence of the 9*E*,11*E* *s*-cisoid conformational restriction in retinoids TTNN (6), SR11254 (13), and SR11262 (14) reduced binding to RAR α . TTNN had about 5-fold higher affinity for RAR γ than for RAR α , whereas SR11254 (13) and SR11262 (14) were unable to displace 50% of bound label from RAR α at concentrations as high as 1 μM , indicating their low affinity for RAR α . In contrast, the IC_{50} s for the RAR α -selective Am580 and Am80 were 2.3 and 88 nM, respectively.

The retinoids that displayed high affinity for RAR α were able to inhibit the anchorage-dependent growth of MCF-7 cells, whereas the two retinoids SR11254 (13) and SR11262 (14), which bound poorly to RAR α even at concentrations as high as 1 μM , were only able to poorly inhibit anchorage-dependent cell growth (<10%) at 12.5 μM ,

which was the highest concentration tested, after 7 days of treatment. Correlation diagrams of the relative binding affinities of these retinoids for RAR α and RAR γ with their inhibition of cell growth under anchorage-dependent growth conditions (Fig. 3) indicated that the ability of retinoids to inhibit adherent cell growth correlated with their affinity for RAR α ($n = 14$, $P \leq 0.001$) but not with their affinity for RAR γ ($n = 14$, $P > 0.1$). Removal of SR11254 (13) and SR11262 (14) from this series still gave a high correlation of growth inhibition with binding to RAR α ($n = 12$, $P < 0.005$) but not to RAR γ ($n = 12$, $P > 0.1$). Both the RAR α -selective retinoids Am580 (7) and Am80 (8) were effective growth inhibitors as were their diazene *N*-oxide isosteres. The two RAR antagonists also inhibited growth, with the higher RAR α affinity retinoid Ro41-5253 (11) being 6-fold more potent as an inhibitor. Ro41-5253 did not show any transcriptional activation activity in MCF-7 cells, as evidenced by the absence of any chloramphenicol acetyl transferase activity on transfection of these cells with the RARE-*tk*-CAT reporter construct (data not shown).

The results were more complex for anchorage-independent growth inhibition by these retinoids. Correlation diagrams did not show a strongly significant correlation between anchorage-independent colony growth inhibition at 1 nM retinoid and the relative retinoid binding affinities to either RAR α ($n = 14$, $y = 12.4427 + 0.4586x$, $r = 0.65$, $P > 0.02$) or RAR γ ($n = 14$, $y = 10.3795 + 0.5714x$, $r = 0.57$, $P > 0.05$). SR11254 (13) and SR11262 (14) were poor inhibitors of anchorage-independent cell growth as evidenced by their IC_{50} s, which were several orders of magnitude higher than those for RA (1), TTAB (5), or Am580 (7). Am580 and its diazene *N*-oxide analogue SR11273 (9) had comparably high inhibitory activity, whereas their respective isomers Am80 (8) and SR11274 (10) were poorer inhibitors. The two antagonists Ro41-5253 (11) and SR3986 (12) were very poor inhibitors of growth ($\leq 10\%$) at 1 nM. A high correlation between RAR binding affinity and the inhibition of anchorage-independent cell growth was only achieved after the two antagonists were removed from the series. Correlation diagrams of the relative RAR binding affinities of the remaining 12 retinoids with their anchorage-independent growth inhibition at 1 nM (Fig. 4) showed an excellent correlation between clonal growth inhibition and binding to RAR α ($n = 12$, $P \leq 0.005$) but not to RAR γ ($n = 12$, $P > 0.05$). The percentage of growth inhibition by the retinoid agonists at 10 nM (data not shown)

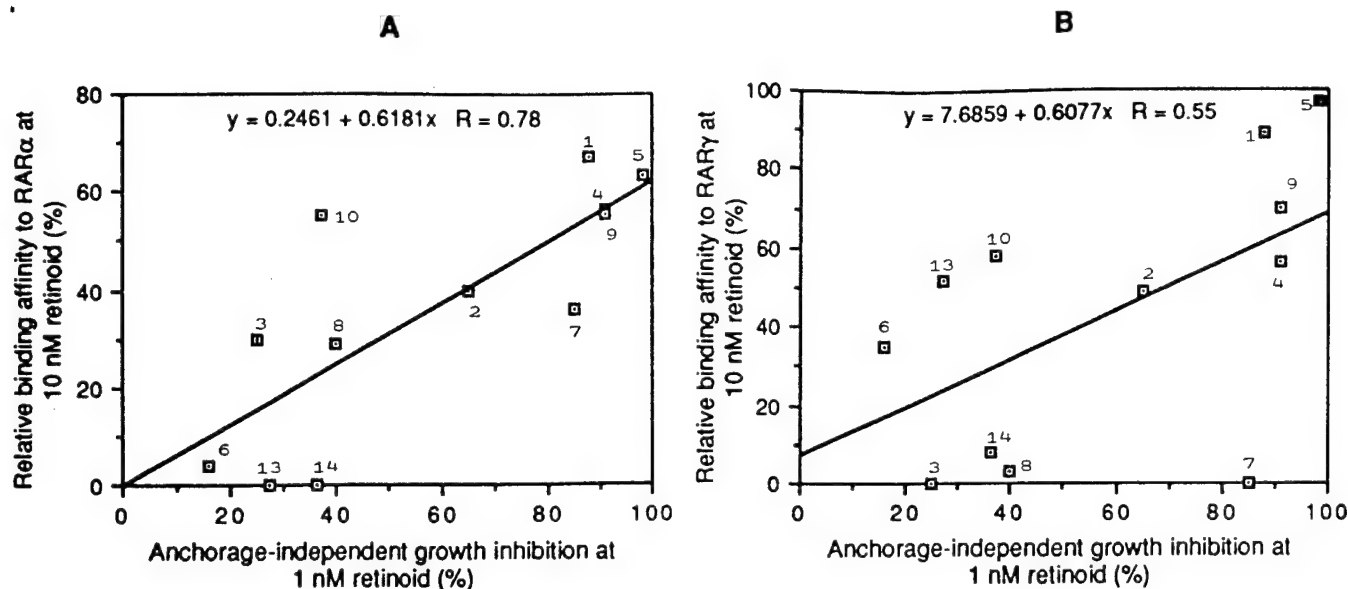


Fig. 4. Binding of retinoid agonists to RAR α and RAR γ compared to their inhibition of MCF-7 mammary carcinoma cell anchorage-independent growth. The relative RAR binding affinities of 12 retinoid agonists, as measured by their ability to displace [3 H]TTAB bound to recombinant human RAR α (A) or human RAR γ (B), were correlated with their inhibition of anchorage-independent cell growth at 1 nM. Clonal proliferation was measured by colony counting after 21 days of retinoid treatment.

also correlated with the binding of these agonists to RAR α ($n = 12$, $r = 0.92$, $P \leq 0.001$) but not to RAR γ ($n = 12$, $r = 0.46$, $P > 0.1$).

DISCUSSION

Correlation studies comparing the binding affinity of these 14 retinoids to their growth-inhibitory activities indicate that of the two receptors present in MCF-7 breast cancer cells, RAR α appears to mediate the RA-induced inhibition of adherent or anchorage-dependent cell growth after 7 days in culture. The two retinoids that did not bind well to RAR α , SR11254 (13) and SR11262 (14), were poor inhibitors of the adherent growth of MCF-7 cells because they retarded growth at 12.5 μ M by only 6 and 9%, respectively, after 7 days. Longer retinoid treatment times led to some growth inhibition by the RAR γ -selective retinoids but also increased the sensitivity of the cells to the RAR α -selective retinoids, as determined by their correspondingly lower IC_{50} s. Growth inhibition did not appear to depend on receptor activation for gene transcription from a RARE, because the two RAR antagonists that did not activate the RARs for gene transcription in a transfection assay were also inhibitory. SR3986 (12), which bound to both RAR α and RAR γ , was a poorer inhibitor than the RAR α -selective antagonist Ro41-5253 (11), which had higher affinity for RAR α . Two major mechanisms of retinoid action have thus far been identified. In the first, gene transcription is stimulated by a retinoid-retinoid receptor complex that activates a RARE located in the promoter region of a gene (6). In the second, gene expression is repressed by a retinoid-retinoid receptor complex that interferes with gene activation from an AP-1 site by the Jun/Fos transcription factor complex (7). Retinoids that inhibit AP-1 activity but lack transcriptional activation activity have been identified previously (35, 36). Therefore, our finding that both retinoid agonists and antagonists are capable of interfering with anchorage-dependent cell growth suggests that their growth inhibitory activity may proceed through an AP-1 pathway.

Anchorage-independent cell growth is indicative of cell tumorigenicity, and its inhibition may be a more meaningful indicator of the chemotherapeutic efficacy of a retinoid. The inhibition of anchorage-independent cell growth only correlated well with binding to RAR α , not with binding to RAR γ , although those retinoids that preferentially

bound to RAR γ were able to inhibit growth at higher concentrations (≥ 5 nM). The RAR antagonists were unable to inhibit clonal growth, which suggests that different inhibitory mechanisms may be involved in the inhibition of anchorage-independent and anchorage-dependent cell growth. The antagonist Ro41-5253 (11) did not interfere with the binding of RAR α to a RARE but, according to proteolysis studies, did confer a different conformation on this receptor than that observed in the presence of RA (22). Therefore, our results also suggest that different conformations of the ligand-binding domain of RAR α may occur in these two different inhibitory processes or that binding of the receptor by the antagonist may directly prevent the binding of a component that is essential for inhibiting anchorage-independent cell growth. The finding that Am80 (8) and its analogue SR11274 (10) were less effective inhibitors of anchorage-independent growth than either Am580 (7) or SR11273 (9) but were comparably effective inhibitors of adherent cell growth further supports our premise that different receptor geometries are required to effect these growth-inhibitory processes.

These studies show the importance of using receptor-selective retinoids as probes to investigate the molecular mechanism of retinoid receptor function and indicate the potential of these compounds as therapeutic agents for selectively affecting cellular processes through interaction with specific retinoid receptors and response elements.

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REFERENCES

- Garcia, M., Derocq, D., Freiss, G., and Rochefort, H. Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. *Proc. Natl. Acad. Sci. USA*, 89: 11538-11542, 1992.
- Guilbaud, N. F., Gas, N., Dupont, M. A., and Valette, A. Effects of differentiation-inducing agents on maturation of human MCF-7 breast cancer cells. *J. Cell Physiol.*, 145: 162-172, 1990.
- Fontana, J. A. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation. *Exp. Cell Biol.*, 55: 136-144, 1987.
- Sheikh, M. S., Shao, Z. M., Chen, J.-C., Hussain, A., Jetten, A. M., and Fontana, J. M. Estrogen receptor-negative breast cancer cells transfected with the estrogen receptor

- exhibit increased RAR- α gene expression and sensitivity to growth inhibition by retinoic acid. *J. Cell Biochem.*, 57: 394-404, 1993.
5. Stunnenberg, H. G. Mechanisms of transactivation by retinoic acid receptors. *Bioessays*, 15: 309-315, 1993.
6. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. The retinoid receptors. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids. Biology, Chemistry and Medicine*, pp. 319-349. New York: Raven Press, 1994.
7. Saatcioglu, F., Helmborg, A., and Karin, M. Modulation of immune function through interference between nuclear receptors and AP-1. *Fundam. Clin. Immunol.*, 2: 27-36, 1994.
8. Allenby, G., Bocquell, M.-T., Saunders, M., Kazmer, S., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P., and Levin, A. A. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc. Natl. Acad. Sci. USA*, 90: 30-34, 1993.
9. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. 9-*cis* Retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell*, 68: 397-406, 1992.
10. Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzel, C., Rosenberger, M., Lovey, A., and Grippo, J. F. 9-*cis* Retinoic acid stereoisomer binds and activates the nuclear receptor RXR α . *Nature (Lond.)*, 355: 359-361, 1992.
11. Roman, S. D., Clarke, C. L., Hall, R. E., Alexander, I. E., and Sutherland, R. L. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.*, 52: 2236-2242, 1992.
12. Shao, Z.-M., Sheikh, M. S., Chen, J.-C., Kute, T., Aisner, S., Schnaper, L., and Fontana, J. A. Expression of retinoic acid nuclear receptors (RARs) and retinoid X receptors (RXRs) in estrogen receptor positive and negative breast cancer. *Int. J. Oncol.*, 4: 849-853, 1994.
13. Roman, S. D., Ormandy, C. J., Manning, D. L., Blaney, R. W., Nicholson, R. L., Sutherland, R. L., and Clarke, C. L. Estradiol induction of retinoic acid receptors in human breast cancer cells. *Cancer Res.*, 53: 5940-5945, 1993.
14. Fontana, J. A., Nervi, C., Shao, Z. M., and Jetten, A. M. Retinoid antagonism of estrogen responsive transforming growth factor α and p52 gene expression in breast carcinoma cells. *Cancer Res.*, 52: 3938-3945, 1992.
15. Dhir, U., Schutt, A., Zhang, X., Tamborini, B., Manjunath, G., Connell, E., Bayona, W., Thomas, M., Rosenberger, M., Truitt, G., Sedarati, F., and Hsu, M. C. Functional acid receptors are essential but not sufficient for *in vitro* anti-proliferative response to retinoids in carcinoma tumor cell lines. *Proc. Am. Assoc. Cancer Res.*, 35: 90, 1994.
16. Urbach, J., and Rando, R. Isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid. *Biochem. J.*, 299: 459-465, 1994.
17. Van der Burg, B., van der Leede, B. M., Kwarkbos-Isbrucker, L., Salverda, S., DeLaat, S. W., and van der Saag, P. T. Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol. Cell. Endocrinol.*, 91: 149-157, 1993.
18. Rudd, C. J., Mansbridge, J. N., Suing, K. D., and Dawson, M. I. Correlation of the ability of retinoids to inhibit promoter-induced anchorage-independent growth of JB6 mouse epidermal cells with their activation of retinoic acid receptor γ . *Cancer Lett.*, 73: 41-49, 1993.
19. Sakashita, A., Kizaki, M., Pakkala, S., Schiller, G., Tsuruoka, N., Tomosaki, R., Cameron, J. F., Dawson, M. I., and Koeffler, H. P. 9-*cis*-Retinoic acid: effects on normal and leukemic hematopoiesis *in vitro*. *Blood*, 81: 1009-1016, 1993.
20. Dawson, M. I., Hobbs, P. D., Chan, R. L., Chao, W., and Fung, V. A. Aromatic retinoic acid analogues: synthesis and pharmacological activity. *J. Med. Chem.*, 23: 583-592, 1981.
21. Loeliger, P., Bollag, W., and Mayer, H. Arotinoids: a new class of highly active retinoids. *Eur. J. Med. Chem.-Chim. Ther.*, 15: 9-15, 1980.
22. Apfel, C., Bauer, F., Cretaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W., and Klaus, M. A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. *Proc. Natl. Acad. Sci. USA*, 89: 7129-7133, 1992.
23. Dawson, M. I., Hobbs, P. D., Derdzinski, K. A., Chao, W., Frenking, G., Loew, G. H., Jetten, A. M., Napoli, J. L., Williams, J. B., Sani, B. P., and Wille, J. J. Effect of structural modifications in the C7-C11 region of the retinoid skeleton on biological activity in a series of aromatic retinoids. *J. Med. Chem.*, 32: 1504-1517, 1989.
24. Dawson, M. I., Chan, R. L., Derdzinski, K., Hobbs, P. D., Chao, W., and Schiff, L. J. Synthesis and pharmacological activity of 6-[(E)-2-(2,6,6-trimethyl-1-cyclohexen-1-yl)ethen-1-yl]- and 6-[(1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-naphthyl)-2-naphthalenecarboxylic acids. *J. Med. Chem.*, 26: 1653-1656, 1983.
25. Kagechika, H., Kawachi, E., Hashimoto, Y., Himi, T., and Shudo, K. Retinobenzonic acids. 1. Structure-activity relationships of aromatic amides with retinoid activity. *J. Med. Chem.*, 31: 2182-2192, 1988.
26. Dawson, M. I., Chao, W.-R., Hobbs, P. D., and Delair, T. The inhibitory effects of retinoids on the induction of ornithine decarboxylase and the promotion of tumors in mouse epidermis. In: M. I. Dawson and W. H. Okamura (eds.), *Chemistry and Biology of Synthetic Retinoids*, pp. 385-466. Boca Raton, FL: CRC Press, Inc., 1990.
27. Dawson, M. I., Hobbs, P. D., Rhee, S. W., Morimoto, H., and Williams, P. G. Synthesis of [1',3',4'-³H₃]-4-(5',6',7',8'-tetrahydro-5',5',8',8'-tetramethyl-2'-anthracenyl)benzoic acid for binding studies of the retinoic acid receptors. *J. Labelled Compd. Radiopharm.*, 33: 633-637, 1993.
28. Kagechika, H., Himi, T., Namikawa, K., Kawachi, E., Hashimoto, Y., and Shudo, K. Retinobenzonic acids. 3. Structure-activity relationships of retinoid azobenzene-4-carboxylic acids and stilbene carboxylic acids. *J. Med. Chem.*, 32: 1098-1108, 1989.
29. Ostrowski, J., Currier, S., Yu, K.-L., Sterzycki, R., Starrett, J., Mansuri, M., and Reczek, P. R. The human retinoic acid receptor γ recognizes a chiral ligand. Mechanism of Action of Retinoids, Vitamin D, and Steroid Hormones, Poster B-25, March 15-20, 1993, Banff, Alberta, Canada.
30. Schiff, L. J., Okamura, W. H., Dawson, M. I., and Hobbs, P. D. Structure-biological activity relationships of new synthetic retinoids on epithelial differentiation of cultured hamster trachea. In: M. I. Dawson and W. H. Okamura (eds.), *Chemistry and Biology of Synthetic Retinoids*, pp. 307-363. Boca Raton, FL: CRC Press, Inc., 1990.
31. Li, X., Shao, Z.-M., Sheikh, M. M., Eisman, J., Jetten, A. M., Chen, J.-C., Dawson, M. I., Aisner, S., Rishi, A., Gutierrez, P., Schnapper, L., and Fontana, J. A. Retinoic acid nuclear receptor β (RAR β) inhibits breast carcinoma growth and tumorigenicity. *J. Cell. Physiol.*, in press, 1995.
32. Quick, T. C., Traish, A. M., and Niles, R. M. Characterization of human retinoic acid receptor α 1 expressed in recombinant baculovirus-infected Sf9 insect cells. *Receptor*, 4: 65-80, 1994.
33. Lombardo, A., Costa, E., Chao, W.-R., Toll, L., Hobbs, P. D., Jong, L., Lee, M.-O., Pfahl, M., Ely, K. R., and Dawson, M. I. Recombinant human retinoic acid receptor β : binding of synthetic retinoids and transcriptional activation. *J. Biol. Chem.*, 269: 7297-7303, 1994.
34. Lehmann, J. M., Dawson, M. I., Hobbs, P. D., Husmann, M., and Pfahl, M. Identification of retinoids with nuclear receptor subtype-selective activities. *Cancer Res.*, 51: 4804-4809, 1991.
35. Fanjul, A., Dawson, M. I., Hobbs, P. D., Jong, L., Cameron, J. F., Harlev, E., Graupner, G., Lu, X.-P., and Pfahl, M. A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature (Lond.)*, 372: 107-111, 1994.
36. Nagpal, S., Athanikar, J., and Chandraratna, R. A. S. Separation of transactivation and AP1 antagonism functions of retinoic acid receptor α . *J. Biol. Chem.*, 270: 923-927, 1995.

Modulation of Retinoic Acid Sensitivity through Dynamic Balance of Orphan Receptors Nur77 and COUP-TF and their Heterodimerization

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Abstract

The diverse function of retinoic acid (RA) is mediated by its nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). However, RA response is often lost in cancer cells that express the receptors. Previously, it was demonstrated that the RA response is regulated by COUP-TF orphan receptor. Here, we present evidence that nur77, another orphan receptor whose expression is highly induced by phorbol esters and growth factors, is involved in modulation of the RA response. Expression of nur77 enhances ligand-independent transactivation of RAREs and desensitizes their RA responsiveness. Conversely, expression of COUP-TF sensitizes RA responsiveness of RAREs by repressing their basal transactivation activity. Unlike the effect of COUP-TFs, the function of nur77 does not require direct binding of nur77 with the RAREs, but is through interaction between nur77 and COUP-TFs. The interaction occurs in solution and results in inhibition of COUP-TF RARE binding and transcriptional activity. In human lung cancer and bladder cancer cell lines, COUP-TF is highly expressed in RA-sensitive cell lines while nur77 expression is associated with RA-resistance of certain cancer cell lines. Stable expression of COUP-TF in nur77-positive, RA-resistant lung cancer cells enhances the efficacy of RA to induce RAR β gene expression and growth inhibition. These observations demonstrate that a dynamic equilibrium between orphan receptors nur77 and COUP-TF, through their heterodimerization that regulates COUP-TF RARE binding, is critical for RA responsiveness of RAREs.

Introduction

Retinoic acid (RA) and its natural and synthetic vitamin A derivatives (retinoids) are known to regulate a broad range of biological processes, and are currently used in the treatment of epithelial cancer and promyelocytic leukemia (Lotan, 1981; Warrell et al., 1993; Gudas et al., 1994; Hong et al., 1994). However, retinoid resistance associated with many different types of cancer has prevented retinoids from further application (Warrell et al., 1993; Hong et al., 1994). The effects of retinoids are mainly mediated by two classes of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Zhang and Pfahl, 1993; Kastner et al., 1995; Mangelsdorf and Evans, 1995). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily that also includes a number of orphan receptors whose ligands and function remain to be determined (Zhang and Pfahl, 1993; Kastner et al., 1995; Mangelsdorf and Evans, 1995). RARs and RXRs modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs). Some of the target genes are RARs themselves, in particular the RAR β gene where a RARE (β RARE) was identified in its promoter region (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). Up-regulation of RAR β gene by RA presumably plays a critical role in amplifying RA response. It correlates with RA-induced growth inhibition in certain types of cancer cell lines in vitro (Li et al., 1996; Liu et al., 1996) and is associated with clinical response in patients with premalignant oral lesions in vivo (Lotan et al., 1995).

Although the expression of RARs and RXRs is essential for RA response, we and others have recently demonstrated that it is not sufficient to render RA target genes responsive to RA (van der Leede et al., 1993; Zhang et al., 1994; Kim et al., 1995; Moghal and Neel, 1995). In lung cancer cell lines, RARs and RXRs are well expressed, but the majority of the cell lines are RA resistant, and RA responsive genes, such as RAR β gene, could not be induced by RA (Zhang et al., 1994). In a lung carcinogenesis model, retinoid receptor function is intact in malignant human bronchial epithelial cells despite their refractory to the growth inhibitory effects of retinoids (Kim et al., 1995). The loss of RAR β induction by RA has been mapped to the β RARE in certain lung cancer cell lines

(Zhang et al., 1994; Moghal and Neel, 1995). However, expression of endogenous RARs and RXRs or cotransfection of RARs and RXRs could not restore RA responsiveness of the β RARE (Zhang et al., 1994; Moghal and Neel, 1995). These observations suggest that RA sensitivity is also influenced by factors other than RARs and RXRs. One of the factors known to regulate RA response is COUP-TF. COUP-TF is encoded by two distinct genes, COUP-TFI (ear-3) (Miyajima et al., 1988; Wang et al., 1989) and COUP-TFII (ARP-1) (Ladiaz and Karathanasis, 1991), that are orphan members of nuclear receptor superfamily (Zhang and Pfahl, 1993; Kastner et al., 1995; Mangelsdorf and Evans, 1995). COUP-TF was originally shown to stimulate gene transcription (Pastorcic et al., 1986). However, subsequent work has demonstrated that COUP-TF can repress transcription induced by a number of nuclear receptors including RARs, thyroid hormone receptors (TRs) and vitamin D receptor (VDR) (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992). Since COUP-TFs bind strongly to response elements of RARs, TRs and VDR and are able to form heterodimers with RXR, the common cofactor of RARs, TRs and VDR, a direct competition for DNA binding site and heterodimerization with RXR have been proposed for this repression (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992). However, results from a recent study (Butler and Parker, 1995) using a two-hybrid system demonstrate that competition of COUP-TF homodimers for DNA binding site is likely the major mechanism for the repression. The binding specificity of COUP-TFs exhibits a strong preference for those bound by retinoid receptors (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992), suggesting that COUP-TFs are involved in the regulation of some RA target genes. More intriguingly, COUP-TF could be activated by dopamine and cAMP-dependent pathways through an yet unidentified indirect mechanism (Power et al., 1991).

Nur77 (also known as NGFI-B and TR3) (Chang and Kokontis, 1988; Hazel et al., 1988; Milbrandt, 1988) is another orphan member of nuclear receptor superfamily. It is rapidly induced by a variety of growth stimuli, including growth factors and phorbol esters (Hazel et al., 1988; Fahrner et al., 1990; Williams and Lau, 1993; Wilson et al., 1993; Lim et al., 1995). Nur77 may play a role

in the regulation of steroidogenic enzyme expression (Wilson et al., 1993; Crawford et al., 1995) and in the control of activation-induced apoptosis of T-cells (Liu et al., 1994; Woronicz et al., 1994). The mechanism by which nur77 exerts its biological functions remains largely unknown. Nur77 may regulate gene expression necessary to alter the cellular phenotype in response to the growth stimuli by binding to its recognition element (NBRE) as a monomer (Wilson et al., 1991). Phosphorylation of nur77 upon induction by growth factors may regulate its DNA binding and transcriptional activity (Fahrner et al., 1990; Hazel et al., 1991; Davis et al., 1993). The NBRE consists of the half-site binding motif (AGGTCA) of RAR/TR/VDR with two additional adenine nucleotides at its 5' end (AAAGGTCA) (Wilson et al., 1991). Interestingly, such sequences are found in the RAR β gene promoter and are located within the β RARE (Perlmann and Jansson, 1995). Investigation of the binding of nur77 on the β RARE demonstrates that nur77 can bind to the β RARE as a heterodimer with RXR (Forman et al., 1995; Perlmann and Jansson, 1995). These observations suggest that nur77 may be involved in the regulation of RAR β gene expression and may function to mediate the interaction between retinoid and growth signalings.

We have recently shown that up-regulation of RAR β expression by RA correlates with RA-induced growth inhibition in human breast cancer and lung cancer cell lines (Li et al., 1996; Liu et al., 1996). In RA-sensitive cancer cell lines, expression of RAR β is strongly induced by RA. In contrast, RA had little effect on RAR β expression in RA-resistant cancer cell lines (Li et al., 1996; Liu et al., 1996). Induction of RAR β by RA is mediated by the β RARE present in its promoter (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). Activation of the β RARE by RA is mainly through RAR/RXR heterodimers (Hermann et al., 1992; Zhang et al., 1992a; Valcarcel et al., 1994). However, we have found that RARs and RXRs are well expressed in some RA-resistant cancer cell lines (Zhang et al., 1994; Li et al., 1996; Liu et al., 1996). In the course to investigate the effect of nur77 on RA-induced RAR β gene expression, we found that nur77 could significantly enhance the transactivation activity of RAREs in a RA- and RARE-binding-independent manner. By using a variety of approaches, we demonstrate that the effect of nur77 is due to inhibition of COUP-TF

RARE binding through direct protein-protein interaction. Transient transfection analysis reveals that COUP-TF RARE binding functions to sensitize RAREs their RA responsiveness and, conversely that *nur77* desensitizes RAREs through its ability to inhibit COUP-TF RARE binding. In human lung cancer and bladder cancer cells, loss of RA sensitivity is associated with overexpression of *nur77* and/or low expression levels of COUP-TF, and can be restored by introduction and expression of COUP-TF. These results reveals a novel regulatory mechanism established through heterodimerization of orphan receptors, that is expected to play a crucial role in the regulation of cellular proliferation, differentiation and carcinogenesis processes, and the cross-talk between growth factor and vitamin A signal transduction pathways.

Results

Nur77 Enhances RARE activity in a RA-independent manner

We have recently shown that induction of RAR β by RA mediates the growth inhibitory effects of retinoids in human breast cancer and lung cancer cells (Liu et al., 1996; Li et al., 1996). RA-induced RAR β expression is mainly mediated by the β RARE in its promoter (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). To investigate the effect of *nur77* on the transactivation of the β RARE, the β RARE cloned into pBLCAT₂ that contains tk promoter linked with CAT gene, the β RARE-tk-CAT (Hoffmann et al., 1990), was used as a reporter gene and transiently transfected into CV-1 cells. When *nur77* expression vector was cotransfected, both all-*trans* RA- and 9-*cis* RA-induced reporter gene activities were enhanced in a concentration dependent manner (Figure 1A). Cotransfection of 200 ng of *nur77* expression vector resulted in about 2-fold increase of the reporter activity when cells were treated with all-*trans* RA. Surprisingly, the basal transcription of the reporter was even greatly increased, with about 5-fold increase. The enhancement was specific to the β RARE because addition of *nur77* did not show any effect on the parental pBLCAT₂ reporter. A similar effect was also observed with a CAT reporter containing the RAR β gene promoter from

-60 to +70, including the β RARE (Hoffmann et al., 1990) (Figure 1A). To investigate the possibility that the nur77 response is due to the presence of a NBRE within the β RARE (Wilson et al., 1991; Perlmann and Jansson 1995), we changed two adenines in the spacing region of the β RARE to mutate the NBRE. The mutations introduced do not affect the consensus half-site binding motifs of RAR/RXR heterodimers. The resulting element ($\Delta\beta$ RARE) was cloned into pBLCAT₂ and used as a reporter. When the reporter was analyzed, we observed a similar increase of their basal transcription by cotransfection of nur77 expression vector (Figure 1A). This observation suggests that the presence of NBRE in the β RARE is not essential for the enhancing effect of nur77. To determine whether the enhancing effects of nur77 could be extended to other hormone response elements, reporter constructs containing TREpal (Glass et al., 1988), ApoAI-RARE (Rottman et al., 1991), CRBPI-RARE (Husmann et al., 1992), Lactoferrin-RARE (Lee et al., 1995), CRBP II-RARE (Mangelsdorf et al., 1991), or a thyroid hormone specific response element (MHC-TRE) (Flink and Morkin 1990) were transfected into CV-1 cells together with or without nur77 expression vector (Figure 1B). Similar to the effect on the β RARE, various degrees of enhancement by nur77 were observed with reporter constructs containing TREpal, ApoAI-RARE, CRBPI-RARE, Lactoferrin-RARE, or CRBP II-RARE (Figure 1B). However, cotransfection of nur77 did not show any effect on the reporter containing MHC-TRE (Figure 1B), suggesting that the effect of nur77 may be specific to RAREs. Thus, nur77 can enhance the transactivation of various RAREs in a RA-independent manner.

The effect of nur77 on RAREs does not require direct nur77-RARE interaction

To investigate whether the enhancement of RARE activity by nur77 is due to its binding to the elements, gel retardation assays were performed. When the β RARE was used as a probe, nur77 alone did not exhibit clear binding (Figure 2A). However, a strong complex was formed with the β RARE when nur77 was mixed with RXR. The complex could be upshifted by anti-nur77 antibody and abolished by anti-RXR antibody, demonstrating that the complex represents RXR/nur77 heterodimers. However, when the $\Delta\beta$ RARE was used as a probe, we did not see any binding of

RXR/nur77 heterodimers (Figure 2B). This data suggests that the integrity of the nur77 binding site (NBRE) within the β RARE is required for efficient RXR/nur77 binding. When the binding of nur77 on other RAREs, such as TREpal, CRBPI-RARE, CRBP II-RARE, HIV-RARE and ApoAI-RARE, was analyzed, we did not observe any binding of nur77 on these elements either in the presence or absence of RXR or RAR, except a weak RXR/nur77 heterodimer binding with the CRBP II-RARE (Figure 2B). As a control, RXR/RAR heterodimers formed a strong complex to all these RAREs. Together, these results indicate that nur77 enhances the activities of different RAREs via a mechanism that is unlikely to involve a direct nur77-RARE interaction.

Nur77 inhibits COUP-TF DNA binding

The above data suggest that nur77 may function to repress the activity of an inhibitor on RAREs, thereby alleviating its inhibition. We then investigated the possibility that nur77 affects the binding of COUP-TFs that are known to bind to and restrict transcription of various RAREs (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992). We first examined the effect of nur77 on COUP-TF binding on the β RARE. COUP-TFI or COUP-TFII formed a strong complex with the β RARE (Figure 3A). However, when nur77 protein was added, the COUP-TF binding complex was inhibited. The inhibition was very efficient in that a one-molar excess amount of nur77 almost completely inhibited the COUP-TF binding, and was specific as a similar amount of RAR had no effect on the binding (Figure 3A). This result suggests that nur77 may interact with COUP-TF, resulting in formation of nur77/COUP-TF heterodimers that can not bind to the β RARE. We then investigated whether this interaction could affect nur77/RXR heterodimer binding on the β RARE. When COUP-TFI or COUP-TFII was incubated with nur77 and RXR, the binding of RXR/nur77 on the β RARE was efficiently inhibited (Figure 3B). A two-molar excess amount of COUP-TFI or COUP-TFII was sufficient to inhibit nur77/RXR heterodimer binding. When larger amount of COUP-TFI or COUP-TFII was used, the nur77/RXR heterodimer binding was completely inhibited and binding of COUP-TF appeared. Thus, nur77 and COUP-TF can interact with each other, resulting in mutual inhibition of their DNA binding. To further study the interaction, the effect of

nur77 on COUP-TF binding on TREpal, CRBPI-RARE, CRBP-II-RARE, and ApoAI-RARE was analyzed (Figure 4). As previously reported (Tran et al., 1992), COUP-TFI or COUP-TFII formed a strong complex with these RAREs. Their binding, however, was inhibited by addition of nur77 protein, similar to that observed on the β RARE (Figure 4). The efficiency of inhibition slightly varies among the response elements and may depend on the affinity of COUP-TF on a particular element. Under the conditions used, a two-molar excess amount of nur77 was sufficient to inhibit COUP-TF binding on most of the RAREs used. However, higher efficiency of inhibition was consistently observed when COUP-TF and nur77 were cotranslated (data not shown), suggesting that prevention of the prompt formation of COUP-TF homodimers in solution (Tran et al., 1992) could enhance its interaction with nur77. These data, together with the observation that nur77 could not affect transcription of MHC-TRE (Figure B) to which COUP-TF can not bind (Tran et al., 1992), demonstrate that inhibition of COUP-TF DNA binding by nur77 is likely responsible for its enhancement of transactivation activity of RAREs.

Interaction of nur77 and COUP-TF in solution

To provide evidence that inhibition of COUP-TF DNA binding by nur77 on RAREs was due to a direct interaction of nur77 and COUP-TF in solution, we first performed an immunoprecipitation assay using anti-nur77 antibody (Figure 5A). When 35 S-labeled COUP-TFI or COUP-TFII was mixed with nur77, each was precipitated by anti-nur77 antibody. The coprecipitation of COUP-TFI or COUP-TFII by anti-nur77 antibody was specific because neither could be precipitated by nonspecific preimmune serum. In addition, incubation of anti-nur77 antibody with peptide used to generate anti-nur77 antibody prevented its precipitation. To further study the interaction, we cloned nur77 cDNA into pGEX-2T expression vector and expressed nur77-glutathione-S-transferase fusion protein in bacteria. The fusion protein was immobilized to glutathione sepharose beads, and mixed with either 35 S-labeled COUP-TFI or COUP-TFII protein. As shown in Figure 5B, labeled COUP-TF protein was specifically bound to nur77-immobilized sepharose beads but not to the control beads, demonstrating the specific interaction. To study whether nur77 and COUP-TF could interact

in vivo, we cloned *nur77* and COUP-TF into yeast expression vectors pGBT9 and pGAD424, respectively. The resulting vectors, pGBT9-*nur77* and pGAD424-COUP-TF, were analyzed for their interaction in vivo by the yeast two-hybrid system (Bartel et al., 1993). Expression of *nur77* and COUP-TF strongly activated the LacZ reporter that contains Gal 4 binding sites, while the parental vectors did not show any activity (Figure 5C). Thus, *nur77* and COUP-TF can interact in intact cells. Together, these data demonstrate that *nur77* can inhibit COUP-TF DNA binding through a direct protein-protein interaction.

Antagonism effect of *nur77* and COUP-TF on modulating RA sensitivity of RAREs

The previous demonstration that COUP-TF can inhibit RA-induced activity was mainly based on transient cotransfection assays where COUP-TF might be overexpressed (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992). We then examined the effect of various concentrations of COUP-TF on the β RARE activity. Cotransfection of larger amounts of COUP-TF expression vector with the β RARE-tk-CAT reporter almost completely inhibited RA-induced reporter activity, consistent with previous results (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992). However, at low concentrations (1, 5, or 10 ng), COUP-TF either did not affect or even slightly enhanced the RA-induced β RARE activity (Figure 6A). At these concentrations, COUP-TF significantly inhibited the basal activity of the reporter, resulting in an increase of RA-dependent fold-induction of the β RARE activity (Figure 6B). Without cotransfection of COUP-TF, a four-fold induction of by RA was seen. However, when 10 ng of COUP-TF expression vector was cotransfected, we observed a fourteen-fold induction of reporter activity in response to RA. These data are in agreement with observations made previously on the peroxisome proliferator responsive element (PPRE) (Baes et al., 1995) and ApoA1-RARE (Windom et al., 1992), where cotransfection of COUP-TF enhanced the hormonal sensitivity of both responsive elements. Thus, COUP-TF, at appropriate concentrations that are likely occur in most of cells, can enhance RA sensitivity of the β RARE. To analyze the effect of *nur77* on COUP-TF activity, we cotransfected *nur77* expression vector together with COUP-TF. As shown in Figure

6A, the inhibition of basal transcription of the β RARE by COUP-TF was completely alleviated when nur77 was cotransfected, resulting in a decrease of RA-dependent fold-induction of the β RARE activity (Figure 6B). These data, together with results shown in Figure 1, demonstrate that nur77 can desensitize RA responsiveness of RAREs by antagonizing COUP-TF DNA binding and transcriptional activity.

Expression of nur77 and COUP-TF in human lung cancer and bladder cancer cell lines

The above observations prompted us to investigate whether expression levels of nur77 and COUP-TF correlate with RA sensitivities observed in various lung cancer (Figure 7A) and bladder cancer (Figure 7B) cell lines. These cancer cell lines displayed various degrees of RA sensitivities in inducing β RARE despite that RARs and RXRs are well expressed in these cell lines (Zhang et al., 1994; data not shown). Although the degree of RA induction of β RARE activity may also depend on levels of retinoid receptors expressed in the cell lines, we found a perfect correlation between COUP-TF expression and RA induction of β RARE activity (Figure 7). COUP-TF was well expressed in Calu-6, H460, H596, SK-MES-1 and H661 lung cancer cell lines, and in J82 and HT-1376 bladder cancer cell lines, in which β RARE activity was highly induced by RA. In contrast, COUP-TF transcripts were not detected in other cancer cell lines that did not show a clear induction of β RARE activity in response to RA. These observations raise the possibility that the expression of COUP-TF in these cancer cell lines might sensitize β RARE responsiveness to RA through its binding to the element. When the expression of nur77 was analyzed, we found that it was highly expressed in RA-resistant H520 and H292 lung cancer cell lines. High levels of nur77 were also observed in RA-sensitive H661, H460 and J82 cell lines. However, these cell lines showed significant amounts of COUP-TF. The observation that COUP-TF and nur77 can antagonize each other's activity suggests that the RA sensitivity in these cell lines may be maintained by the expression of COUP-TF that can counteract nur77 effect. Under conditions used, we did not detect

expression of *nur77* in RA-resistant H441, SCaBER and 5637 cell lines. It is likely that factors other than *nur77* may be responsible for RA resistant in these cells.

Dynamic balance of *nur77* and COUP-TF regulates RA sensitivity in human cancer cell lines

In our previous studies, we observed that RAR β was differentially expressed in several human lung cancer cell lines (Zhang et al., 1994). RAR β was not expressed in Calu-6 lung cancer cells but its expression was greatly induced by RA treatment. In contrast, RAR β was highly expressed in H292 lung cancer cells but in a RA-independent manner (Figure 8A). The observations that COUP-TF is expressed in RA-sensitive Calu-6 but not in RA-resistant H292 cells and that *nur77* is expressed in H292 but not in Calu-6 cells (Figure 7) suggest that relative expression levels of COUP-TF and *nur77* may affect expression of RARE-containing genes, such as RAR β . We, therefore, analyzed whether different RA sensitivities of β RARE in Calu-6 and H292 cells could be attributed to levels of *nur77* and COUP-TF in the cells. We first investigated the effect of *nur77* in RA-sensitive Calu-6 cells (Figure 8B). When *nur77* expression vector was cotransfected together with the β RARE-tk-CAT into the cells, we observed an increase of basal activity and a decrease of RA-dependent fold-induction of the reporter. Cotransfection of 50 ng of *nur77* expression vector reduced RA-dependent β RARE activity from seven-fold to two-fold. We next analyzed the effect of COUP-TF on RA-resistant H292 cells (Figure 8C). Cotransfection of COUP-TF expression vector with the β RARE-tk-CAT into the cells reduced basal reporter activity while RA-induced activity was not clearly affected. In the absence of COUP-TF, we did not see clear effect of RA on β RARE activity. However, when 20 ng of COUP-TF expression vector was cotransfected, we found a three-fold induction of the β RARE activity by RA. Thus, a dynamic balance of *nur77* and COUP-TF is important in regulating RA sensitivity of the β RARE in these cancer cells and that overexpression of *nur77* and/or lack of COUP-TF may be responsible for RA-resistance in H292 cells.

Stable expression of COUP-TF restores RA sensitivity in RA resistant human lung cancer cells

The observations that nur77 and COUP-TF are differentially expressed in RA-sensitive Calu-6 and RA-resistant H292 cells (Figure 7) and that they can antagonize each other's transcriptional activity (Figure 8) suggest a possibility that constitutive expression of RAR β in H292 cells may be due to overexpression of nur77 and lack of COUP-TF in the cells. To test whether expression of COUP-TF could antagonize the effect of nur77 and sensitize RAR β expression responsiveness to RA in H292 cells, we stably expressed COUP-TF in the cells. Two stable clones (H292/COUP-TFI-2 and H292/COUP-TFI-3) that expressed COUP-TF were subject to analysis of their RAR β gene expression in the absence or presence of RA. In the absence of RA, levels of RAR β expression in these stable clones was much lower than that in the parental H292 cells (Figure 9A). Surprisingly, treatment of the stable clones with RA strongly enhanced RAR β gene expression in the stable clones while it had little effect on the parental H292 cells (Figure 9A). Most importantly, we observed that RA, that was not able to inhibit the growth of parental H292 cells, could now strongly inhibit the growth of the stable clones, with about 85% of inhibition observed when the cells were treated with 10^{-6} M RA for six days (Figure 9B). Thus, the expression of COUP-TF could sensitize RA responsiveness of RAR β expression and growth inhibition in RA-resistant H292 lung cancer cells.

Discussion

The diverse function of RA is mainly mediated by RARs and RXRs. However, expression of RARs and RXRs is not sufficient to render cells RA responsiveness. This becomes especially apparent in certain RA-resistant cancer cells (Zhang et al., 1994; Kim et al., 1995; Moghal and Neel, 1995). Here we provide evidence that orphan receptors COUP-TF and nur77 play a critical role in the regulation of RA responsiveness of various RA target genes through their modulation of RARE binding. COUP-TFs bind to a variety of RAREs and sensitize their RA responsiveness.

Conversely, nur77 reduces RA sensitivity of RAREs through heterodimerization with COUP-TF, that results in inhibition of COUP-TF binding to RAREs. These observations demonstrate important role of nuclear orphan receptors in the regulation of RA responses. It is expected that many of the orphan receptors may function to modulate the response of ligand-activated receptors and their response elements.

COUP-TFs function to sensitize RA responsiveness of RAREs

Results from several previous studies demonstrate that COUP-TFs function as negative regulators of RA target genes (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Windom et al., 1992). Several lines of evidence presented here suggest that COUP-TFs may also function to sensitize the RA responsiveness of certain RA target genes. In transient transfection assays in CV-1 (Figure 6) and in lung cancer cells (Figure 8), expression of appropriate amounts of COUP-TF repressed the basal transcription of the β RARE-tk-CAT reporter while it had no effect on RA-induced reporter activity. Such an effect results in an increase of RA sensitivity of the β RARE (Figures 6 and 8). Similar effect of COUP-TF was also observed on the peroxisome proliferator responsive element (Baes et al., 1995). When we analyzed RARE binding of nuclear proteins prepared from RA-sensitive and RA-resistant lung cancer cell lines, we observed strong RARE-binding complexes in RA-sensitive cells but not in RA-resistant cells. Using anti-COUP-TF antibody, we found that the major complex observed in RA-sensitive cells represents COUP-TF binding (data not shown). These results, together with the observation that COUP-TF binds to RAREs in vitro (Figures 3 and 4), suggest that the repressive effect of COUP-TF on β RARE basal activity is due to its binding to the element, that prevents RAREs from binding and activating by other RA-independent activators. One of the well-known factor that binds to the β RARE is MB67 (Baes et al., 1994). MB67 is another orphan member of nuclear hormone receptor superfamily. It binds to the β RARE as a heterodimerize with RXR, and activates the β RARE in a RA-independent manner (Baes et al., 1994). Thus, it is expected that, in the absence of COUP-TF, the β RARE may be activated by MB67 or similar activators in a RA-independent manner. Other constitutive activators on RAREs

have been also identified. In the case of DR-1 type of RAREs, HNF-4, a liver enriched nuclear orphan receptor (Sladek et al., 1990), binds to the element and acts as a strong constitutive transcriptional activator (Jiang et al., 1995). The observations that COUP-TFs bind to this type of RAREs and antagonize HNF-4 effect (Garcia et al., 1993) suggest that COUP-TFs may also function to protect these RAREs from binding and activating by ligand-independent activators.

Our finding that, at appropriate concentrations, COUP-TF does not inhibit RA-induced RARE activity (Figures 6 and 8) implies that the binding of COUP-TF on RAREs may be replaced by retinoid receptors in the presence of RA. This would suggest that retinoid receptors, upon binding to RA, have higher affinity to RAREs. We showed previously that binding of RXR homodimers to RAREs was promoted by its ligand 9-cis RA in vitro (Zhang et al., 1992b). Although RA does not show any effect on RAR/RXR heterodimer DNA binding in vitro (Zhang et al., 1992a; Zhang et al., 1992b), a recent study, using in vivo footprinting, demonstrates that RAR ligands can promote receptor β RARE binding in vivo (Chen et al., 1996). Thus, it is likely that, in vivo, ligand induces conformational changes of retinoid receptors so that they have higher affinity to RARE, that would allow them to replace COUP-TF RARE binding, and subsequently RA responses. Thus, one of the main functions of COUP-TF is to protect certain RAREs from RA-independent activation and thereby maintain the RA sensitivity of the RAREs once RA is available. Thus, COUP-TFs may act to sensitize certain RAREs their RA responsiveness while at the same time function as negative regulators of other RAREs. Whether they function as sensitizers or negative regulators may largely depend on the relative affinity of COUP-TFs to RAREs and their cellular concentrations. At certain concentrations, COUP-TF may sensitize certain RAREs their responsiveness to RA. The RAREs, such as the β RARE, have relative low affinity to COUP-TFs (Tran et al., 1992) so that the binding of COUP-TFs can be competed and replaced by liganded-retinoid receptors. In contrast, the affinity of COUP-TFs to some other RAREs may be higher and their binding can not be replaced by liganded-retinoid receptors. COUP-TFs may therefore function as negative regulators of these RAREs.

Nur77 modulates RARE activity through interaction with COUP-TF

Nur77 is an immediate-early protein whose expression is rapidly induced by a variety of growth stimuli (Hazel et al., 1988; Fahrner et al., 1990; Williams and Lau 1993; Wilson et al., 1993; Lim et al., 1995). It has been reported that nur77 binds its recognition element (NBRE) as monomer to regulate expression of its target genes (Wilson et al., 1991). However, it was reported that nur77 could form heterodimers with RXR on β RARE (Forman et al., 1995; Perlmann and Jansson, 1995), suggesting that nur77 is also involved in the regulation of retinoid function. In present study, we show that nur77 can enhance transactivation activity of a variety of RAREs (Figure 1). Nur77 alone or in the presence of RAR or RXR did not show binding to the RAREs except the β RARE (Figure 3A), indicating that DNA binding is not involved in nur77-mediated activation. In the case of the β RARE, binding of nur77/RXR heterodimers may contribute to its effect since we have observed that cotransfection of RXR augments the enhancing effect of nur77 on the β RARE (data not shown). These findings prompted us to investigate the interaction of nur77 and COUP-TF. Our gel retardation assay shows effective inhibition of COUP-TF binding by nur77 (Figures 3 and 4). The inhibition of COUP-TF binding is conferred through a direct nur77-COUP-TF interaction in solution as revealed by our immunoprecipitation (Figure 4A) and GST-pull down assays (Figure 4B). Furthermore, the interaction occurs in vivo (Figure 4C). In transient transfection assay, nur77 can counteract the effect of co-transfected COUP-TF in CV-1 cells (Figure 6) and in lung cancer cells (Figure 8), resulting in reduced RA sensitivity of RARE. These observations demonstrate that nur77 is involved in the regulation of RA sensitivity of various RAREs. This is supported by our finding that nur77 is highly expressed in certain RA-resistant cancer cell lines, such as H292 and H520 cells (Figure 7). Since COUP-TFs are known to bind to certain vitamin D and thyroid hormone response elements (Cooney et al., 1992; Kliwer et al., 1992; Tran et al., 1992; Windom et al., 1992), our finding that nur77 can interact with COUP-TFs suggest that nur77 may be also involved in the regulation of other hormonal signalings.

Regulation of RA responsiveness of RAR β expression

Up-regulation of RAR β expression by RA presumably plays an important role in amplifying RA response and has been shown to be associated with RA-induced growth inhibition in cancer cells (Li et al., 1996; Liu et al., 1996) and clinical retinoid response in patients with premalignant oral lesions (Lotan et al., 1995). Loss of such mechanism has been observed in certain RA-resistant cancer cells (Zhang et al., 1994; Li et al., 1996; Liu et al., 1996). Induction of RAR β by RA is mainly mediated by the β RARE in its promoter (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). We show that nur77 and COUP-TF can reciprocally repress one another's β RARE binding (Figure 3). This observation and transient transfection assays in CV-1 (Figure 6) and in lung cancer cells (Figure 8) as well as stable transfection assay in H292 lung cancer cells (Figure 9) provide convincing evidence that RA sensitivity of β RARE is largely controlled by relative concentrations of COUP-TF and nur77 providing that retinoid receptors are expressed. In our study of human lung cancer and bladder cancer cell lines, we show a close correlation between relative abundance of COUP-TF and nur77 and RA sensitivity of the β RARE in the majority of cancer cell lines (Figure 7). Nur77 is highly expressed in certain RA-resistant cancer cell lines, suggesting that overexpression of nur77 may be responsible for loss of β RARE sensitivity to RA in certain cancer cell lines, such as H292 cells. In contrast, COUP-TF is only expressed in cancer cell lines in which the β RARE is highly sensitive to RA. When COUP-TF was stably introduced and expressed in RA-resistant H292 lung cancer cells, the RA-independent expression of RAR β gained RA-dependency. These observations indicate that COUP-TFs do not function as negative regulators on the β RARE and suggest that appropriate concentrations of COUP-TFs are required for RA to induce RAR β expression and subsequently the growth inhibition (Figure 9). This is reminiscent of in vivo observations (Reuberte et al., 1993; Lutz et al., 1994) that RAR β expression is restricted to motor neurons at the time when COUP-TF is highly expressed.

Biological significance of nur77-COUP-TF interaction

The findings described here further demonstrate the extensive cross-talk between two major signal transduction pathways that modulate gene transcription in response to vitamin A, phorbol esters and growth factors. Previous discoveries (Nicholson et al., 1990; Schule et al., 1991; Yang-Yen et al., 1991) have demonstrated that the two systems interact through interaction between retinoid receptor and AP-1, that appears to be mediated by CREB-binding protein (CBP) (Kamei et al., 1996). The present findings reveal a novel mechanism that mediates the cross-talk through interaction between nur77 and COUP-TF, that modulates RA sensitivity of RAREs. We show that RA sensitivity of the RARE is largely modulated by COUP-TF and nur77. We have found that modulation of nur77 expression levels in cancer cells by changing fetal calf serum concentration in the culture medium and/or treatment of phorbol ester TPA dramatically affects RA sensitivity of the cells (data not shown), demonstrating that nur77 mediates the effect of growth factors and phorbol esters on retinoid response. Nur77 is one of the few nuclear receptors whose expression is rapidly induced by growth factors and cAMP-dependent pathway (Hazel et al., 1988; Fahrner et al., 1990; Williams and Lau 1993; Wilson et al., 1993; Lim et al., 1995). In addition, nur77 can be activated through phosphorylation (Fahrner et al., 1991; Hazel et al., 1991; Davis et al., 1993). Thus, heterodimerization between nur77 and COUP-TF may allow interaction between growth factor and retinoid signalings. Remarkably, COUP-TF has been shown to be activated by dopamine and cAMP-dependent pathways (Power et al., 1991). It remains to be seen whether nur77 mediates the effect of dopamine on COUP-TF activities.

A dynamic equilibrium of the two orphan receptors will likely play a crucial role in the regulation of RA sensitivity program during development and in adult life. It may provide an environment that modulates RA sensitivity of RA target genes during proliferation and differentiation processes. Such environment appears to be well controlled by different cellular stimuli as the expression of nur77 is induced by growth signalings (Hazel et al., 1988; Fahrner et al., 1990; Williams and Lau 1993; Wilson et al., 1993; Lim et al., 1995) while the expression of COUP-TF can be enhanced by RA (Jonk et al., 1994). Loss of such control mechanisms may result in overexpression of nur77 and/or

lack of COUP-TF as seen in H292 and H520 cells. This, in turn, may be responsible for RA resistance, and may contribute to cell proliferation and neoplastic transformation by releasing the inhibitory effect of RA on cell growth. Our demonstration that expression of COUP-TF in RA resistant H292 cells could enhance their RA response provides novel approaches for restoring RA sensitivity in certain RA-resistant cancer cells.

Materials and methods

Cell culture

CV-1 cells were grown in DME medium supplemented with 10% fetal calf serum (FCS). Calu-6, SK-MES-1, J82, HT-1376 and SCaBER cells were maintained in MEM medium supplemented with 10% FCS. H292, H520, H460, H596, H441, H661 and 5631 cells were grown in RPMI1640 supplemented with 10% FCS. A-549 cells were maintained in F12 medium supplemented with 10% FCS.

Plasmids and receptor proteins.

Nur77 expression vector was constructed by cloning the nur77 cDNA (Chang and Kokontis, 1988) fragment into pECE vector. The construction of the reporter plasmids β RARE-tk-CAT, TREpal-tk-CAT, CRBPI-RARE-tk-CAT, CRBPII-RARE-tk-CAT, Apo AI-RARE-tk-CAT, lactoferrin-RARE-tk-CAT, MHC-TRE-HC-CAT has been described previously (Hermann et al., 1992; Tran et al., 1992; Zhang et al., 1992a; Zhang et al., 1992b; Lee et al., 1995). The reporter $\Delta\beta$ RARE-tk-CAT was obtained by inserting one copy of mutated β RARE oligonucleotide (TGTAGGGTTCACACTGAGTTCACTCA) (underline indicates the mutated nucleotides) into the BamHI site of pBLCAT₂ (Luckow and Schutz, 1987). The RAR β promoter (SmaI-EcoRI fragment) reporter has been described (Hoffmann et al., 1990). The construction of COUP-TFI cDNA into pRc/CMV vector (Invitrogene, San Diego, CA) followed the procedure described

previously (Liu et al., 1996). Receptor proteins were synthesized by in vitro transcription/translation system using rabbit reticulocyte lysate (Promega) as described previously (Pfahl et al., 1990). The relative amount of the translated proteins was determined by ^{35}S -methionine-labeled protein on SDS-PAGE, quantitating the amount of incorporated radioactivity, and normalizing it relative to the content of methionine in each protein.

Transient and stable transfection assay.

For CV-1 cells, they were plated at 1×10^5 cells per well in a 24-well plate 16-24 h before transfection as described previously (Pfahl et al., 1990). For Calu-6 and H292 cells, 5×10^5 cells were seeded in six-well culture plate. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (Pfahl et al., 1990). Briefly, 100 ng of reporter plasmid, 150 ng β -galactosidase expression vector (pCH 110, Pharmacia), and various amounts of nur77 expression vector were mixed with carrier DNA (pBluescript) to 1,000 ng of total DNA per well. CAT activity was normalized for transfection efficiency to the corresponding β -gal activity. For stable transfection, the pRc/CMV-COUP-TFI recombinant plasmid was stably transfected into H292 cells using calcium phosphate precipitation method, and screened using G418 (GIBCO BRL, Grand Island, NY) as described (Liu et al., 1996).

Gel retardation assay.

Gel retardation assay using in vitro synthesized proteins has been described previously (Pfahl et al., 1990). When interaction of nur77 and COUP-TF was studied, they were incubated on ice for 10 min before performing gel retardation in order to prevent the readily formation of COUP-TF homodimer. In most cases, cotranslation of nur77 and COUP-TF resulted in much more efficient dimerization of the two proteins. When antibodies were used in the gel retardation assay, 1 μl anti-nur77 (Santa Cruz Biotech., Inc., Santa Cruz, CA) or 1 μl anti-RXR (Lee et al., 1995) was incubated with receptor protein at room temperature for 30 min prior to performing gel retardation

assay. Oligonucleotides used for gel retardation assay have been described elsewhere (Hermann et al., 1992; Tran et al., 1992; Zhang et al., 1992a; Zhang et al., 1992b; Lee et al., 1995).

Immunoprecipitation assay

For immunoprecipitation assay (Zhang et al., 1992a), 5 μ l reticulocyte lysate containing in vitro translated 35 S-labeled COUP-TFI or COUP-TFII were incubated with 20 μ l in vitro translated nur77 in 100 μ l buffer containing 50 mM KCl and 10% glycerol for 15 min on ice. The reactions were then incubated with 5 μ l anti-nur77 antibody or nonspecific preimmune serum for 2 h on ice. When the peptide from which anti-nur77 antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA) was generated was used, anti-nur77 antibody was incubated with 5 μ l of peptide at room temperature for 30 min before adding to the reaction mixtures. Immunocomplexes were precipitated by adding 40 μ l protein-A-sepharose slurry and mixing continuously in the cold room for 1 h. The complexes were then washed five times with RIPA buffer, resuspended in SDS sample buffer containing 15% β -mercaptoethanol, boiled, and resolved by SDS-PAGE.

GST-pull down assay

To prepare glutathione-S-transferase-nur77 fusion protein (GST-nur77), the nur77 cDNA was cloned in frame into the expression vector pGEX-2T (Pharmacia). The fusion protein was expressed in bacteria using the procedure provided by the manufacturer, and was analyzed by gel retardation assay and western blot (data not shown). To analyze the interaction between nur77 and COUP-TF, the fusion protein was immobilized to glutathion-sepharose beads. For control, the vector protein (glutathione transferase) prepared under the same conditions was also immobilized. The beads were preincubated with BSA (1 mg/ml) at room temperature for 5 min. 35 S-labeled in vitro synthesized receptor proteins (2 to 5 μ l, depending on translation efficiency) were then added to the beads. The beads were then continuously rocked for 1 h at 4°C in a final volume of 200 μ l in EBC buffer (140 mM NaCl, 0.5% NP40, 100 mM NaF, 200 μ M Sodium Orthovanadate, and 50 mM Tris, pH8.0).

After five times washing with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% NP40), the bound proteins were analyzed by SDS-PAGE.

Two-hybrid assay.

For the yeast two-hybrid assay, the yeast two-hybrid system from Clontech Inc. (Palo Alto, CA) was used. Nur77 cDNA was cloned into the yeast expression vector pGBT9 to generate an in-frame fusion with Gal4 DNA binding domain. COUP-TF cDNA was cloned into pGAD424 to produce an in-frame fusion with Gal4 activation domain. The yeast reporter strain Y190 containing a LacZ reporter plasmid with Gal4 binding site was used for transformation. β -galactosidase activity was determined following the conditions provided by the manufacturer to assess the interaction between nur77 and COUP-TF.

MTT assay

To determine the effect of all-*trans* RA on the viability of the stable transfectants, cells were seeded at 1,000 per well in 96-well plate, and treated with various concentrations of all-*trans* RA for six days. Media were changed every 48 h. The number of viable cells were determined by MTT assay as described previously (Mosmann, 1983).

Northern blot

For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method as described (Zhang et al., 1994). 30 μ g of total RNAs from different cell lines treated with or without 10^{-6} M all-*trans* RA were analyzed by Northern blot. RAR β , COUP-TFI or nur77 cDNA was used as a probe. To determine that equal amounts of RNA were used, the expression of β -actin was studied.

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References

- Baes, M., Gulick, T., Choi, H-S., Martinoli, M.G., Simha, D. and Moore, D.D. (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol. Cell. Biol.*, 14, 1544-1552.
- Baes, M., Castelein, H., Desmet, L. and Declercq, P.E. (1995) Antagonism of COUP-TF and PPAR α /RXR α on the activation of the malic enzyme gene promoter: modulation by 9-cis RA. *Biochem. Biophys. Res. Commu.*, 215, 338-345.
- Bartel, P.L., Chien, C.-T., Sternglanz, R. and Fields, S. (1993) Using the two-hybrid system to detect protein-protein interactions. In *Cellular Interactions in Development: A Practical Approach*, Hartley, D.A. (ed.), Oxford University Press, Oxford, pp 153-179.
- Butler, A.J. and Parker, M.G. (1995) COUP-TF II homodimers are formed in preference to heterodimers with RXR α or TR β in intact cells. *Nucl. Acids Res.*, 23, 4143-4150.
- Chang, C. and Kokontis, J. (1988) Identification of a new member of the steroid receptor superfamily by cloning and sequence analysis. *Biochem. Biophys. Res. Commun.*, 155, 971-977.
- Chen, J-Y., Clifford, J., Zusi, C., Starrett, J., Tortolani, D., Ostrowski, J., Reczek, P.R., Chambon, P. and Gronemeyer, H. (1996) Two distinct events in retinoid receptor heterodimer action revealed by ligand synergism. *Nature* (in press).
- Cooney, A.J., Tsai, S.Y., O'Malley, B.W., Tsai, M.-J. (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTC A response elements, allowing COUP-TF to repress hormonal induction of the vitamin D₃ thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.*, 12, 4153-4163.
- Crawford, P.A., Sadovsky, Y., Woodson, K., Lee, S.L. and Milbrandt, J. (1995) Adrenocortical function and regulation of the steroid 21-hydroxylase gene in NGFI-B-deficient mice. *Mol. Cell. Biol.*, 15, 4331-4336.
- Davis, I.J., Hazel, T.G., Chen, R-H., Blenis, J. and Lau, L.F. (1993) Functional domains and phosphorylation of the orphan receptor nur77. *Mol. Endocrinol.*, 7, 953-964.
- de The, H., Vivanco-Ruiz, M.M., Tiollais, P., Stunnenberg, H., and Dejean, A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature*, 343, 177-180.
- Fahrner, T.J., Carroll, S.L., and Milbrandt, J. (1990) The NGFI-B protein, an inducible member of the thyroid/steroid receptor family, is rapidly modified posttranslationally. *Mol. Cell. Biol.*, 10, 6454-6459.

- Forman, B.M., Umesono, K., Chen, J., and Evans, R.M. (1995) Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell*, 81, 541-550.
- Garcia, A.D., Ostapchuk, P and Hearing, P. (1993) Functional interaction of nuclear factors EF-C, HNF-4, and RXR α with hepatitis B virus enhancer I. *Mol. Cell. Biol.*, 67, 3940-3950.
- Glass, C.K., Holloway, J.M., Devary, O.V. and Rosenfeld, M.G. (1988) The thyroid hormone receptor binds opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell*, 54, 313-323.
- Gudas, L.J., Sporn, M.B., and Roberts, A.B. (1994) Cellular biology and biochemistry of the retinoids. In M.B. Sporn, A. B. Roberts, and D.S. Goodman (eds.), *The Retinoids*. 2nd edition., Raven Press. New York, pp. 443-520.
- Hazel, T.G., Nathans, D., and Lau, L.F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* , 85, 8444-8448.
- Hazel, T.G., Misra, R., Davis, I.J., Greenberg, M.E. and Lau, L.F. (1991) Nur77, a growth factor-inducible member of the steroid hormone receptor superfamily, is differentially modified in PC12 cells upon growth factor treatment and membrane depolarization. *Mol. Cell. Biol.*, 11, 3239-3246.
- Hermann, T., Hoffmann, B., Zhang, X-k., Tran, P., and Pfahl, M. (1992). Heterodimeric receptor complexes determine 3,5,3'-triiodothyronine and retinoid signaling specificities. *Mol. Endocrinol.* 6, 1153-1162.
- Hoffmann, B., Lehmann, J.M., Zhang, X-k., Hermann, T., Graupner, G., and Pfahl, M. (1990) A retinoic acid receptor-specific element controls the retinoic acid receptor- β promoter. *Mol. Endocrinol.*, 4, 734-1743.
- Hong, W.K. and Itri, L.M. (1994) Retinoids and human cancer. In Sporn, M.B., Roberts, A.B. and Goodman, D.S. (ed.). *The Retinoids*. Raven Press, New York. p.597-630.
- Husmann, M., Hoffmann, B., Stump, D.G., Shytil, F., and Pfahl, M. (1992) A retinoid acid response element from the rat CRBPI promoter is activated by an RAR/RXR heterodimer. *Biochem. Biophys. Res. Comm.*, 187, 1558-1564.
- Flink, I.L. and Morkin, E. (1990) Interaction of thyroid hormone receptors with strong and weak cis-acting elements in the human α -myosin heavy chain gene promoter. *J. Biol. Chem.*, 265, 11233-11237.
- Jiang, G., Nepomuceno, L., Hopkins, K. and Sladek, F.M. (1995) Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Mol. Cell. Biol.*, 15, 5131-5143.
- Jonk, L.J.C., de Jonge, M.E., Pals, C.E.G.M., Wissink, S., Vervaart, J.M., Schoorlemmer, J., and Kruijer, W. (1994) Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. *Mech. Develop.*, 47, 81-87.
- Kastner, P., Mark, M., and Chambon, P. (1995) Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell*, 83, 859-869.

- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B. Lin, S.-C., Heyman, R.A., Rose, D.W., Glass, C.K., Rosenfeld, M.G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, 85, 403-414.
- Kim, Y-H., Dohi, D.F., Han, G. R., Zou, C-P., Oridate. N., Walsh, G.L., Nesbitt, J.C., Xu, X-C., Hong, W.K., Lotan, L., and Kurie, J.M. (1995) Retinoid refractoriness occurs during lung carcinogenesis despite functional retinoid receptors. *Cancer Res.*, 55, 5603-5610.
- Kliwer, S.A., Kazuhiko, U., Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A. and Evans, R.M. (1992) Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc. Natl. Acad. Sci. USA*, 89, 1448-52.
- Ladiaz, J.A., and Karathanasis, S.K. (1991) Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. *Science*, 251, 561-565.
- Lee, M.O., Hobbs, P.D., Zhang, X.-k., Dawson, M.I. and Pfahl, M. (1994) A synthetic retinoid antagonist inhibits the human immunodeficiency virus type 1 promoter. *Proc. Natl. Acad. Sci. USA*, 91, 5632-36.
- Lee, M.-O., Liu, Y., and X.-k. Zhang. A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. *Mol. Cell. Biol.* 15:4194-4207, 1995.
- Li, Y., Dawson, M.I., Lee, M.-O., Jong, L., Hobbs, P.D. and Zhang, X.-k. (1996) Induction of apoptosis by retinoids in human lung cancer cell lines. (Submitted).
- Lim, R.W., Zhu, C.Y., and Stringer, B. (1995) Differential regulation of primary response gene expression in skeletal muscle cell through multiple signal transduction pathways. *Biochim. Biophys. Acta*, 1266, 91-100.
- Lippman, S.M., Kessler, J.F., and Meyskens, F.L. (1987) Retinoids as preventive and therapeutic anticancer agents. *Cancer Treat. Rep.*, 71, 493-405 (part 1); 493-515 (part 2).
- Liu, Y., Lee, M-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J.C. and Zhang, X.-k. (1996) RAR β mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.*, 16, 1138-1149.
- Liu, Z.-G., Smith, S.W., McLaughlin, K.A., Schwartz, L.M., and Osborne, B.A. (1994) Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene *nur77*. *Nature*, 367, 281-284.
- Lotan, R. (1981) Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochem. Biophys. Acta.*, 605, 33-91.
- Lotan, R., Xu, X.-C., Lippman, S.M., Ro, J.Y., Lee, J.S., Lee, J.J., and Hong, W.K. (1995) Suppression of retinoic acid receptor- β in premalignant oral lesions and its up-regulation by isotretinoin. *N. Engl. J. Med.*, 332, 1405-1410.
- Luckow, B., and Schutz, G. (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res.*, 15, 5490.

- Lutz, B., Kuratani, S., Cooney, A.J., Wawersik, S., Tsai, S.Y., Eichele, G. and Tsai, M-J. (1994) Developmental regulation of the orphan receptor COUP-TFII gene in spinal motor neurons. *Development*, 120, 25-36.
- Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. (1991) A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell*, 66, 555-561.
- Mangelsdorf, D.J., and Evans, R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, 83, 841-850.
- Milbrandt, J. (1988) Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron*, 1, 183-188.
- Miyajima, N., Kadowaki, Y., Fukushige, S., Shimizu, S., Semba, K., Yamanashi, Y., Matsubara, K., Toyoshima, K., and Yamamoto, T. (1988) Identification of two novel members of erb A superfamily by molecular cloning: the gene products of the two are highly related to each other. *Nucl.Acids Res.*, 16, 11057-11074.
- Moghal, N. and Neel, B.G. (1995) Evidence for impaired retinoic acid receptor-thyroid hormone receptor AF-2 cofactor activity in human lung cancer. *Mol. Cell. Biol.*, 15, 3945-3959.
- Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.*, 65, 55.
- Nicholson, R., Mader, S., Nagpal, S., Leid, M., Rochette-Egly, C., and Chambon, P. (1990) Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site. *EMBO J.* 9, 4443-4454.
- Pastorcic, M., Wang, H., Elbrecht, A., Tsai, S.Y., Tsai, M-J. and O'Malley, B.W. (1986) Control of transcription initiation in vitro requires binding of a transcriptional factor to the distal promoter of the ovalbumin gene. *Mol. Cell. Biol.*, 6, 2784-2791.
- Perlmann, T., and Jansson, L. (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurrl. *Genes Dev.*, 9, 769-782.
- Pfahl, M. Tzukerman, M., Zhang, X-k., Lehmann, J.M., Hermann, T., Wills, K.N. and Graupner, G. (1990) Rapid procedure for nuclear retinoic acid receptor cloning and their analysis. *Methods Enzymol.*, 189, 256-270.
- Power, R.F., Lydon, J.P., Conneely, O.M., and O'Malley, B.W. (1991) Dopamine activation of an orphan of the steroid receptor superfamily. *Science*, 252, 1546-1548.
- Reuberte, E., Friederich, V., Chambon, P. & Morriss-Kay, G. (1993) Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. *Development*, 118, 267-282.
- Rottman, J.N., Windom, R.L., Nadal-Ginard, B., Mahdavi, V., and Karathanasis, S.K. (1991) A retinoic acid-responsive element in the apolipoprotein A1 gene distinguishes between two different retinoic acid response pathways. *Mol. Cell. Biol.* 11, 3814-3820.
- Schule, R., Rangarajan, P., Yang, N., Kliewer, s., Ransone, L.J., Bolado, J., Verma, I.M., and Evans, R.M. (1991) Retinoic acid is a negative regulator of AP-1 responsive genes. *Proc. Natl. Acad. Sci. USA*, 88, 6092-6096.

- Sladek, F.M., Zhong, W., Lai, E. and Darnell, J.E. Jr. (1990) Liver-enriched transcriptional factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.*, 4, 2353-2365.
- Sucov, H.M., Murakami, K.K., and Evans, R.M. (1990) Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc. Natl. Acad. Sci. USA*, 87, 5392-5396.
- Tran, P., Zhang, X.-k., Salbert, G., Hermann, T., Lehmann, J.M., and Pfahl, M. (1992) COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol. Cell. Biol.*, 12, 4666-4676.
- Valcarcel, R., Holz, H., Jimenez, C.G., Baretino, D. and Stunnenberg, H.G. (1994) Retinoid-dependent in vitro transcription mediated by the RXR/RAR heterodimer. *Genes & Dev.*, 8, 3068-3079.
- van der leede, B.M., van den Brink, C.E., and van der Saag, P.T. (1993) Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines. *Mol. Carcinog.*, 8, 112-122.
- Wang, L.-H., Tsai, S.Y., Cook, R.G., Beattie, W.G., Tsai, M.-J., and O'Malley, B.W. 1989 COUP transcription factor is a member of the steroid receptor superfamily. *Nature*, 340, 163-166.
- Warrell, R.P. Jr., De The, H., Wang, Z.-Y., and Degos, L. (1993) Acute Promyelocytic Leukemia. *N. Eng. J. Med.*, 329, 177-189.
- Widom, R.L., Rhee, M., Karathansis, S.K. (1992) Repression by ARP-1 sensitizes apolipoprotein AI gene responsiveness to RXR α and retinoic acid. *Mol Cell. Biol.*, 12, 3380-3389.
- Wilson, T.E., Fahrner, T.J., Johnston, M., and Milbrandt, J. (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science*, 252, 1296-1300.
- Williams, G.T., and Lau, L.F. (1993) Activation of the inducible orphan receptor gene *nur77* by serum growth factors: dissociation of immediate-early and delayed-early responses. *Mol. Cell. Biol.*, 13, 6124-6136.
- Wilson, T.E., Mouw, A.R., Weaver, C.A., Milbrandt, J., and Parker, K.L. (1993) The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Mol. Cell. Biol.*, 13, 861-868.
- Woronicz, J.D., Cainan, B. Ngo, V., and Winoto, Astar (1994) Requirement for the orphan steroid receptor *nur77* in apoptosis of T-cell hybridomas. *Nature*, 367, 277-281.
- Yang-Yen, H.F., Zhang, X.-k., Graupner, G., Tzukerman, M., Sakamoto, B., Karin, M., and Pfahl, M. (1991) Antagonism between retinoic acid receptors and AP-1: Implications for tumor promotion and inflammation. *New Biol.*, 3, 1206-1219.
- Zhang, X.-k., Hoffmann, B., Tran, P., Graupner, G., and Pfahl, M. (1992a) Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature*, 355, 441-446.

- Zhang, X-k., Lehmann, J., Hoffmann, B., Dawson, M., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992b) Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature*, 358, 587-591.
- Zhang, X-k. and Pfahl, M. (1993) Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.*, 4, 156-162.
- Zhang, X.-k., Liu, Y., Lee, M-O., and Pfahl, M. (1994) A specific defect in the retinoic acid receptor associated with human lung cancer cell lines. *Cancer Res.*, 54, 5663-5669.

Figure Legends

Figure 1. RA-independent enhancement of RARE activities by nur77. A. Nur77 promotes β RARE and RAR β promoter activities. B. Nur77 activities on various RAREs and TRE. CV-1 cells were transfected with 100 ng indicated CAT reporter gene together with the indicated amounts of nur77 expression vector. Cells were treated with either all-*trans* RA (stripped bar), 9-*cis* RA (dotted bar), thyroid hormone T₃ (open bar), or no hormone (filled bar), and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding β -gal activity. Data shown represent the means of three independent experiments.

Figure 2. Binding of nur77 on various RAREs. A. Nur77 forms heterodimers with RXR on the β RARE. Equal amount of in vitro synthesized nur77 and RXR was incubated alone or together at room temperature for 10 min. The reaction mixtures were then incubated with ³²P-labeled β RARE and analyzed by gel retardation assay. When antibody (Ab) was used, it was incubated with receptor protein for 30 min at room temperature before performing the gel retardation assay. B. Analysis of nur77 binding on various RAREs in the presence or absence of RAR or RXR. An equal amount of nur77 was incubated alone or together with RAR or RXR prior to performing the gel retardation assay using the indicated RARE as a probe. For comparison, the binding of RAR/RXR heterodimers was shown.

Figure 3. Mutual inhibition of nur77 and COUP-TF DNA binding. A. Inhibition of COUP-TF DNA binding on the β RARE by nur77. In vitro synthesized COUP-TF was preincubated with the indicated molar excess of nur77. Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. Following this preincubation, the reaction mixtures were incubated with ³²P-labeled β RARE and analyzed by the gel retardation assay. For the control,

COUP-TF was also preincubated with the indicated molar excess of in vitro synthesized RAR α protein. **B.** Inhibition of nur77/RXR heterodimer binding on the β RARE by COUP-TFs. In vitro synthesized nur77 protein was preincubated with RXR α in the presence or absence of indicated molar excess of COUP-TFI or COUP-TFII, and analyzed by gel retardation assay using the β RARE as a probe. Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction.

Figure 4. Inhibition of COUP-TF binding by nur77 on different RAREs. In vitro synthesized COUP-TF protein was preincubated with the indicated molar excess of nur77 and analyzed by gel retardation assay using the indicated RARE as a probe. Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction.

Figure 5. Direct interaction of nur77 and COUP-TF in solution. **A.** Analysis of nur77/COUP-TF interaction by the immunoprecipitation assay. 35 S-labeled in vitro synthesized COUP-TFI or COUP-TFII was incubated with in vitro synthesized nur77. After incubation, either anti-nur77 antibody or nonspecific preimmune serum (NI) was added. In the control, anti-nur77 antibody was preincubated with a peptide from which the antibody was generated. The immune complexes were washed, boiled in SDS sample buffer and separated on a 10% SDS-PAGE. The labeled COUP-TFI and COUP-TFII are shown for comparison. **B.** Analysis of nur77/COUP-TF interaction by the GST-pull down assay. To further analyze the interaction between nur77 and COUP-TFs, nur77 protein was synthesized in bacteria using pGEX-2T expression vector (Pharmacia). The glutathione-S-transferase-nur77 (GST-nur77) fusion protein were immobilized to the glutathione Sepharose beads. As a control, the same amount of glutathione transferase was also immobilized to the beads. 35 S-labeled COUP-TFI or COUP-TFII was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins were shown for comparison.

C. Analysis of *nur77* /COUP-TF interaction by the yeast two-hybrid assay. The *nur77* and COUP-TFI cDNAs were cloned into the yeast expression vectors pGBT9 and pGAD424, respectively. β -galactosidase activity was assayed from a yeast strain Y190 containing the lacZ reporter plasmid to study the *in vivo* interaction. The β -galactosidase activity measured with the parental yeast expression vectors is shown for control.

Figure 6. Modulation of RA sensitivity of β RARE by COUP-TF and *nur77*. A. β RARE-tk-CAT was cotransfected with the indicated amounts of *nur77* and/or COUP-TF into CV-1 cells. Cells were treated with or without 10^{-7} M all-*trans* RA, and 24 h later assayed for CAT activity. Data shown represent the means of three independent experiments. B. The same data were plotted to indicate the fold-activation by RA.

Figure 7. Expression of COUP-TF and *nur77* and β RARE activity in human lung cancer and bladder cancer lines. A. Expression of COUP-TF and *nur77*, and RA-dependent β RARE activity in human lung cancer cell lines. B. Expression of COUP-TF and *nur77*, and RA-dependent β RARE activity in human bladder cancer cell lines. Total RNAs were prepared from the indicated human lung cancer and bladder cancer cell lines treated with or without 10^{-6} M all-*trans* RA for 24 h and analyzed for the expression of COUP-TF and *nur77*. For control, the expression of β -actin is shown. β RARE activity represents fold-induction by all-*trans* RA as determined by transient transfection assay using the β RARE-tk-CAT as a reporter.

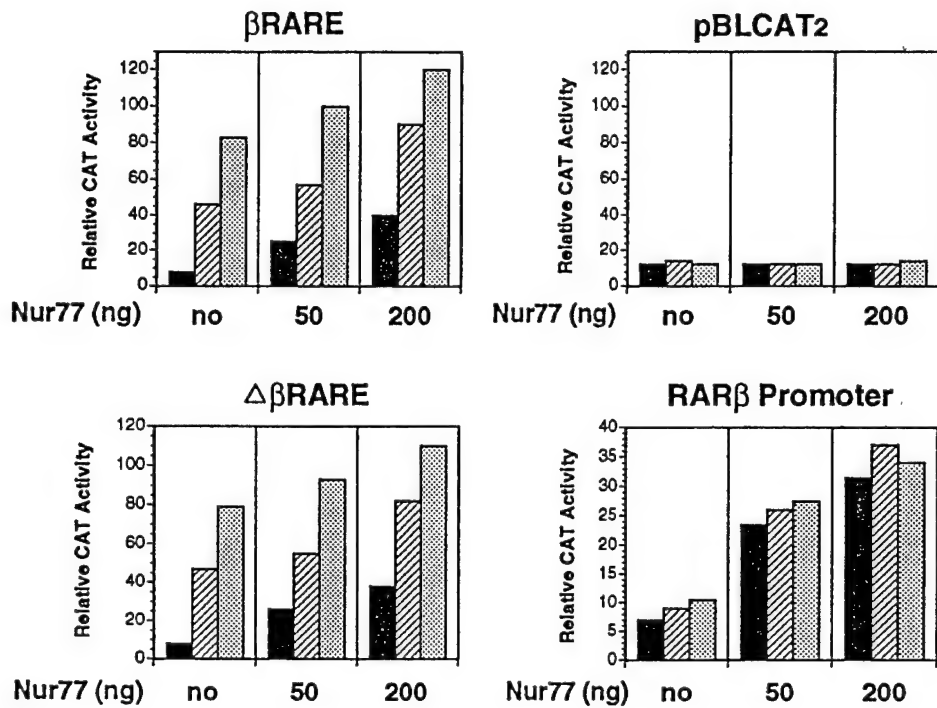
Figure 8. Modulation of RA sensitivity by COUP-TF and *nur77* in human lung cancer cell lines. A. Effect of RA in inducing RAR β expression in Calu-6 and H292 cell lines. Total RNAs were prepared from Calu-6 or H292 lung cancer cells treated with or without 10^{-6} M all-

trans RA for 24 h and analyzed for the expression of RAR β . For control, the expression of β -actin is shown. **B.** Nur77 decreases RA sensitivity in Calu-6 cells. β RARE-tk- CAT was cotransfected with the indicated amounts of COUP-TFI into Calu-6 cells. The cells were treated with or without 10^{-7} M all-*trans* RA for 24 h, and assayed for CAT activity. Data shown represents the means of two experiments. The same data were also plotted to indicate the fold-induction by RA. **C.** COUP-TF enhances RA sensitivity in H292 cells, β RARE-tk-CAT was cotransfected with the indicated amounts of COUP-TFI into H292 cells. The cells were then treated with or without 10^{-7} M all-*trans* RA for 24 h and assayed for CAT activity. The same data were also plotted to indicate the fold-induction by RA. Data shown is the representative of four independent experiments.

Figure 9. Stable expression of COUP-TF in RA-resistant H292 cells restores their RA sensitivity. **A.** Expression of RAR β gene in H292 and stable clones. Total RNAs were prepared from Calu-6 and H292 human lung cancer cell lines treated with or without 10^{-6} M all-*trans* RA for 24 h and analyzed for the expression of RAR β . In the control, the expression of β -actin is shown. **B.** RA-induced growth inhibition in H292 and H292 stable clones that expressed transfected COUP-TF growth inhibition, cells were seeded at 1,000 per well in 96-well plate and treated with the indicated concentrations of all-*trans* RA for six days. The numbers of viable cells were determined by the MTT assay.

Figure 1
Wu et al 1996

A.



B.

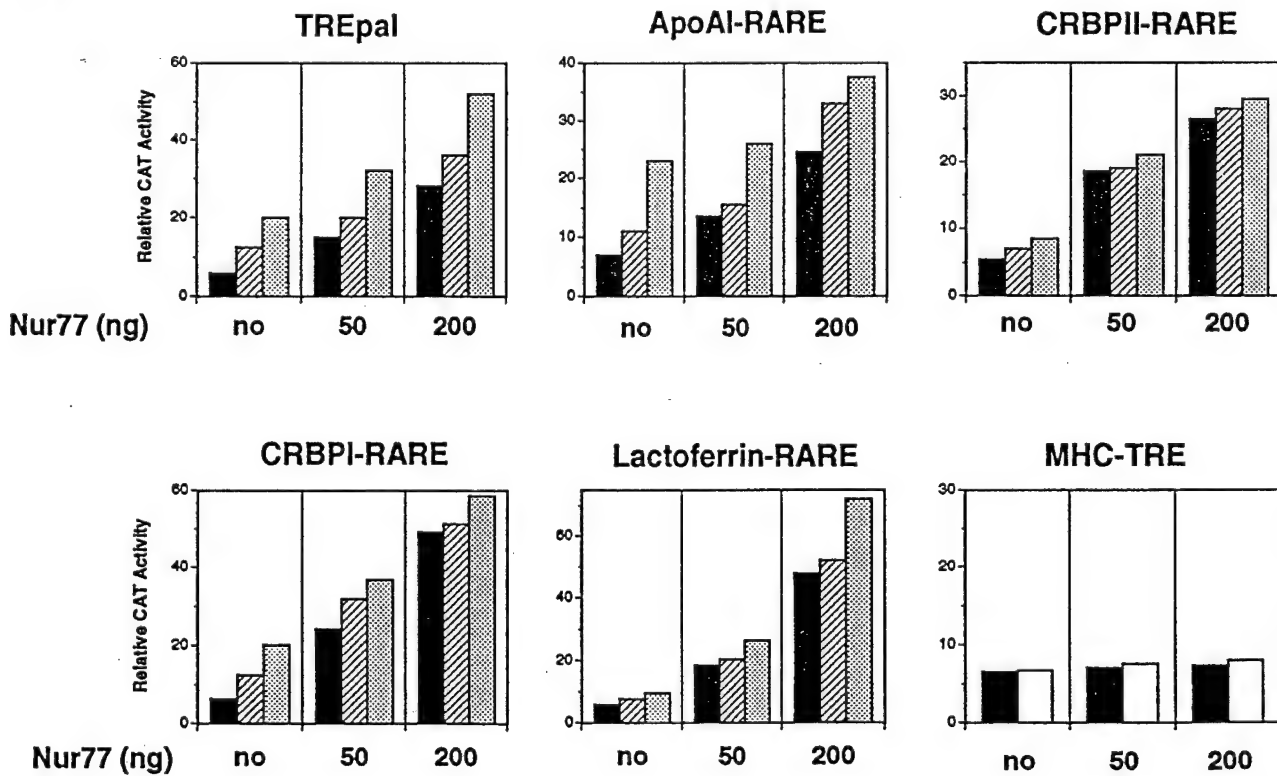


Figure 2A
Wu et al., 1996

A.



Figure 2B
Wu et al., 1996

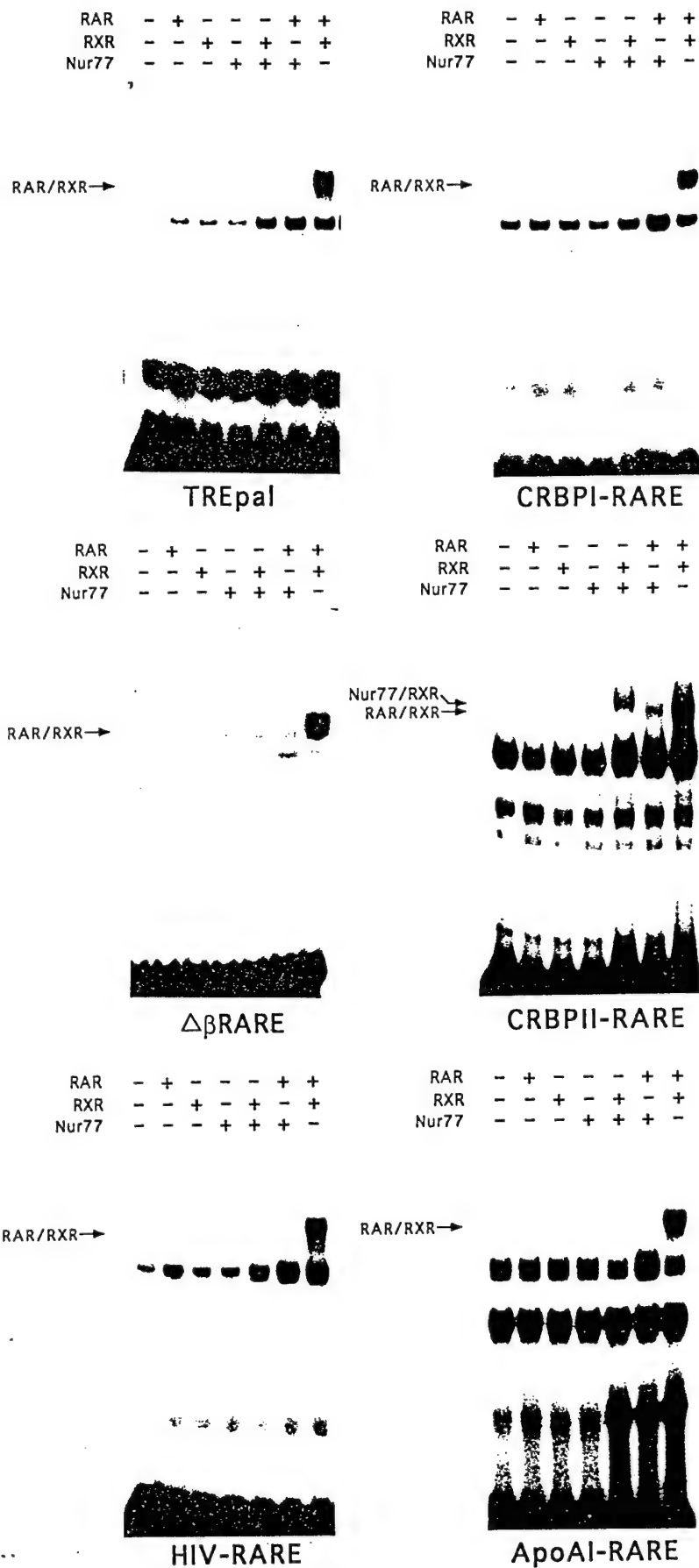
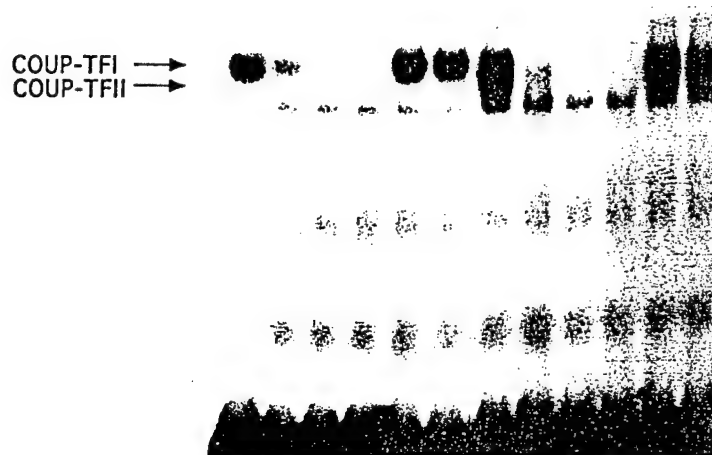


Figure 3
Wu et al 1996

A.

| | | | | | | | | | | | | |
|-----------|---|---|---|---|---|---|---|---|---|---|---|---|
| RAR | - | - | - | - | 1 | 3 | - | - | - | - | 1 | 3 |
| Nur77 | - | 1 | 2 | 3 | - | - | - | 1 | 2 | 3 | - | - |
| COUP-TFII | - | - | - | - | - | - | + | + | + | + | + | + |
| COUP-TFI | + | + | + | + | + | + | - | - | - | - | - | - |



B.

| | | | | | | | | | |
|-----------|---|---|---|---|---|---|---|---|---|
| COUP-TFII | - | - | - | - | - | 1 | 2 | 3 | 4 |
| COUP-TFI | - | 1 | 2 | 3 | 4 | - | - | - | - |
| Nur77/RXR | + | + | + | + | + | + | + | + | + |

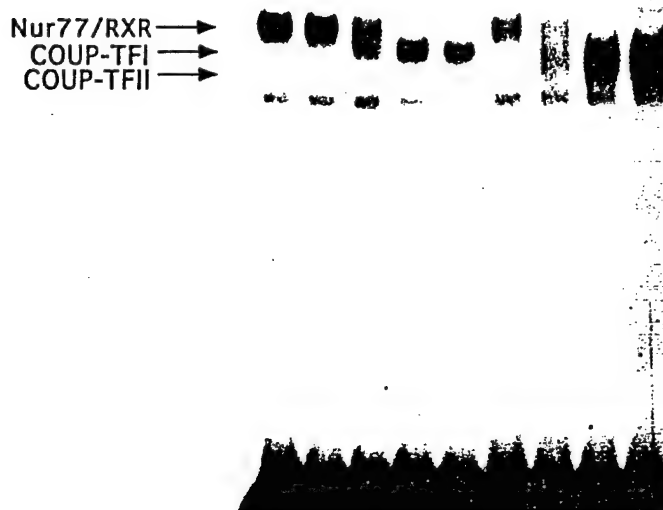


Figure 4
Wu et al 1996

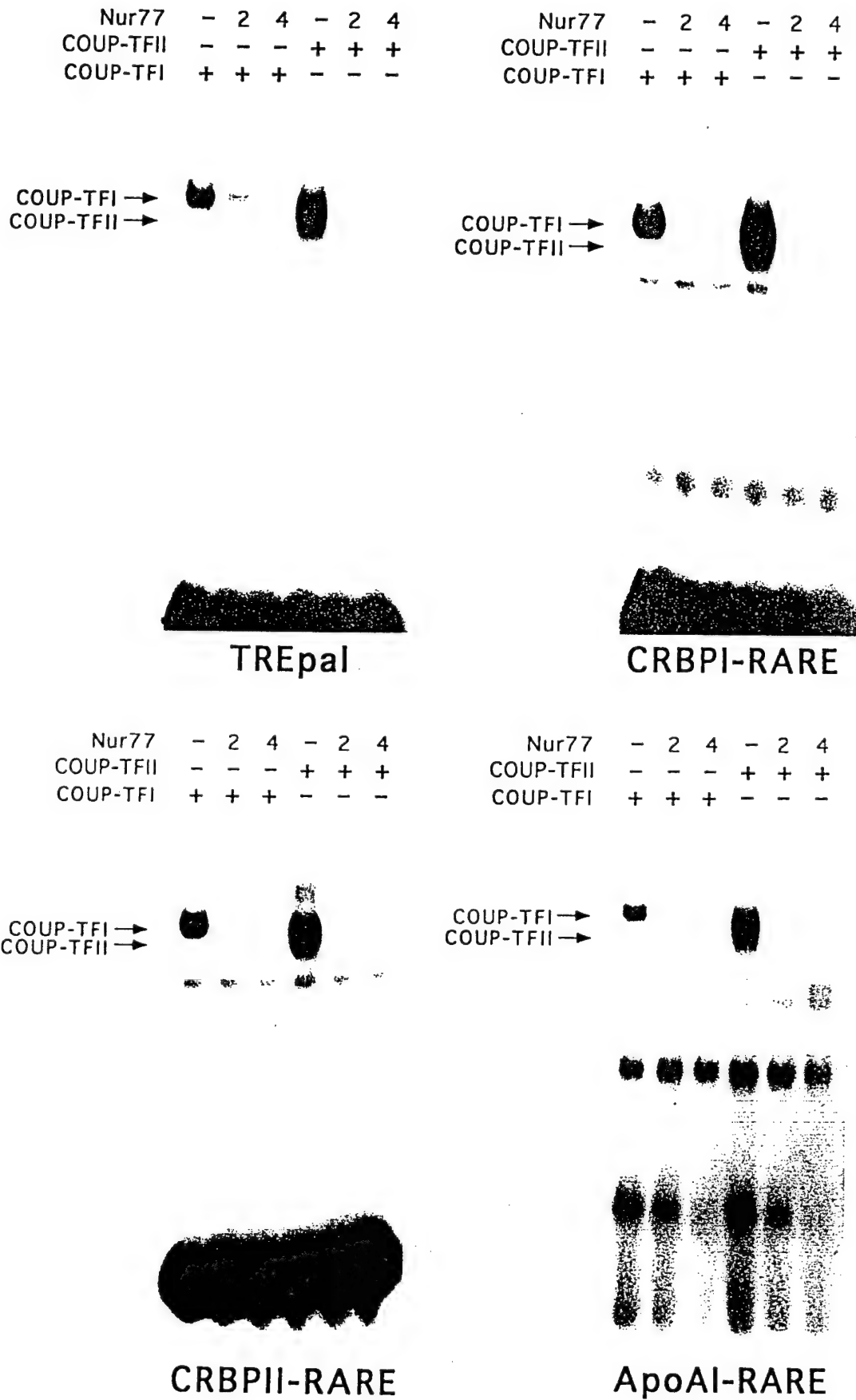
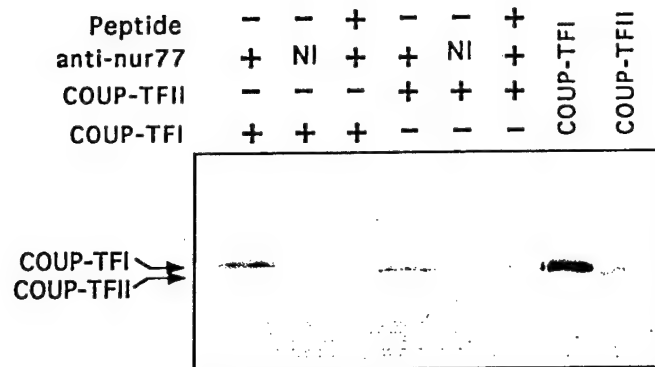
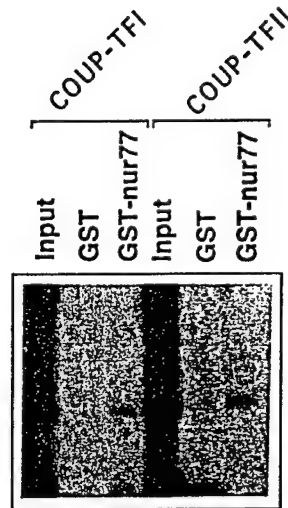


Figure 5
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A.



B.



C.

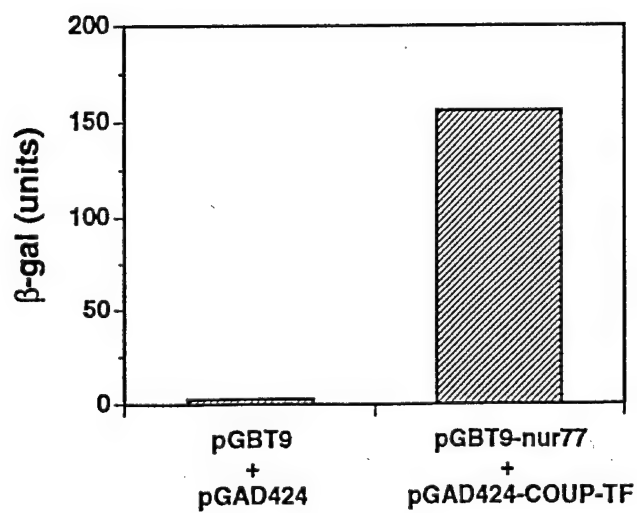
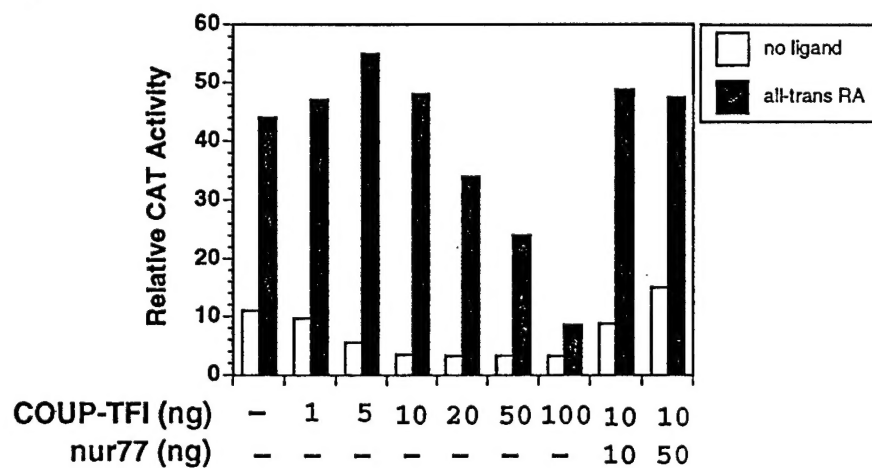


Figure 6
Wu et al 1996

A.



B.

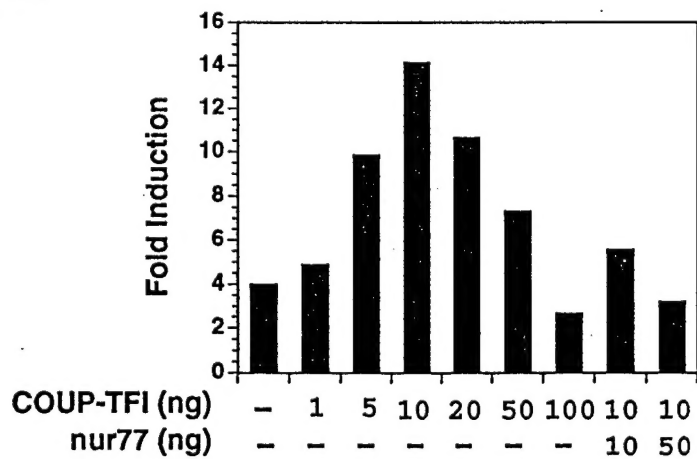
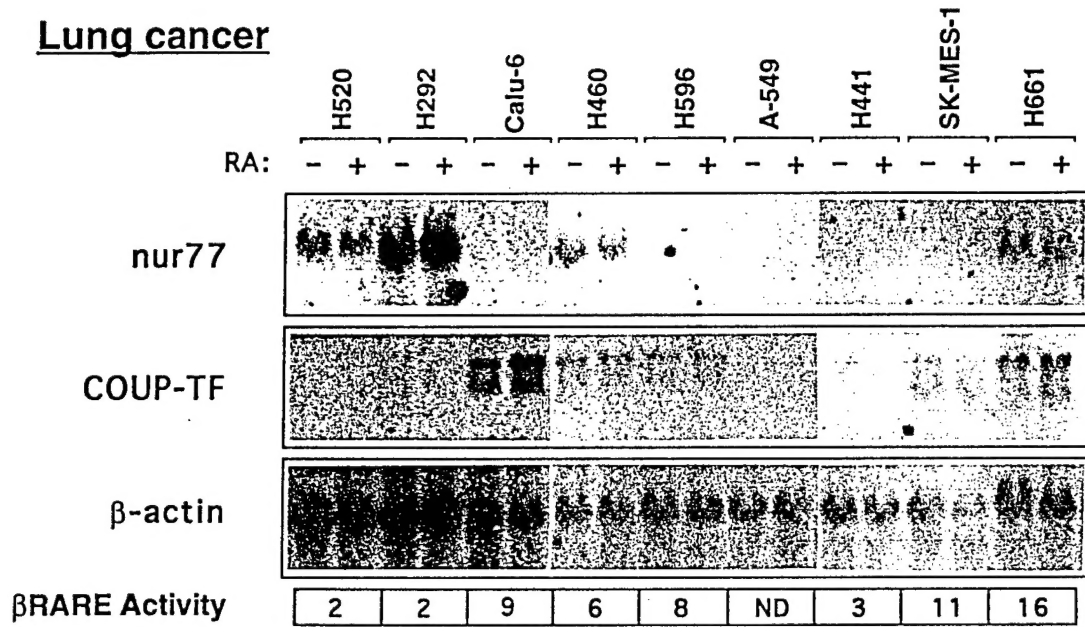


Figure 7
Wu et al 1996

A.



B.

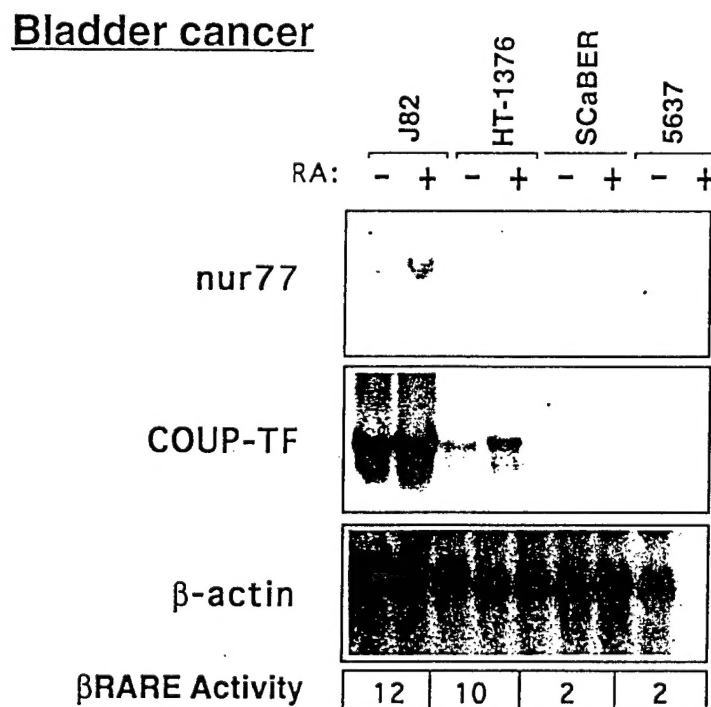


Figure 8
Wu et al 1996

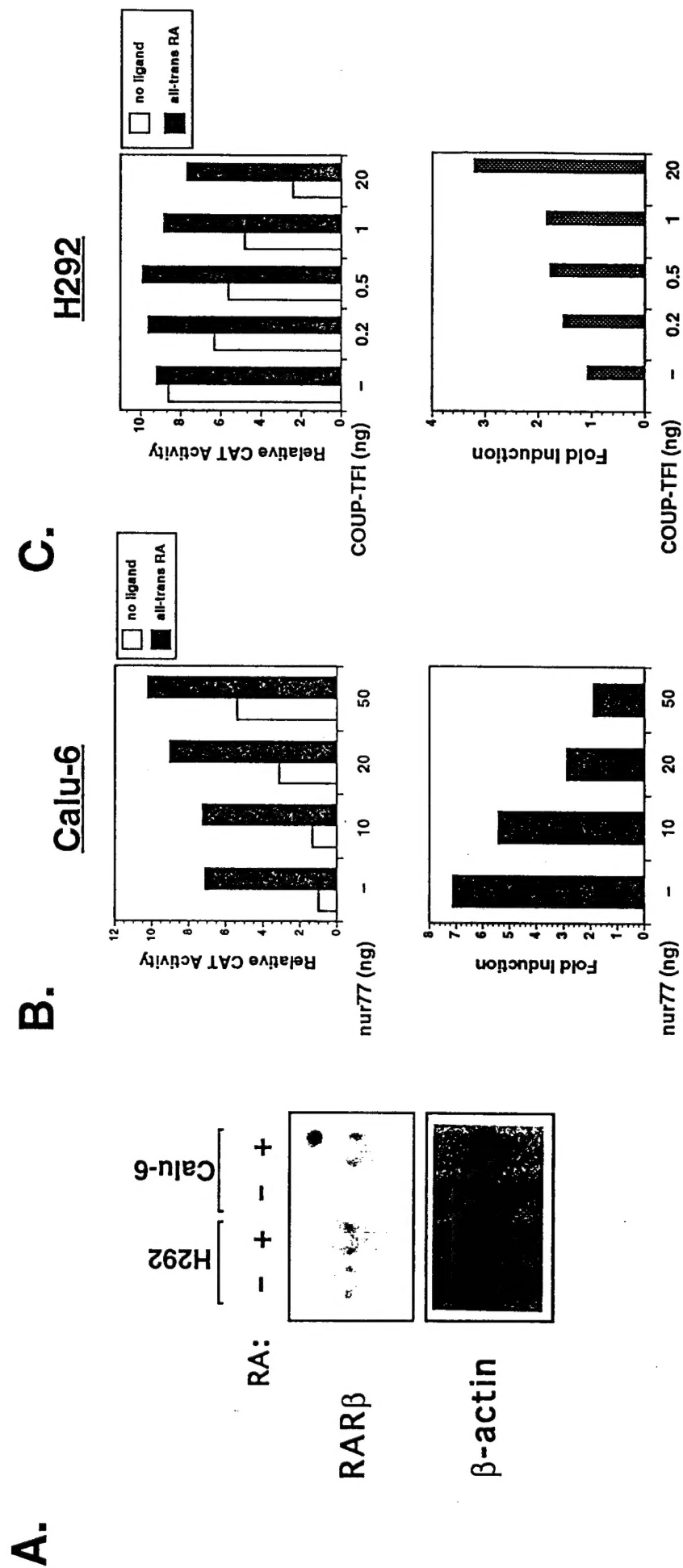
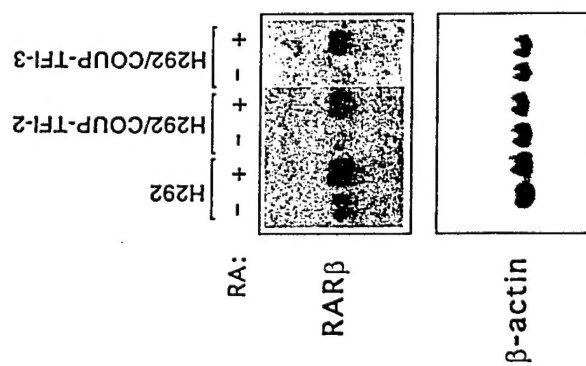


Figure 9
Wu et al 1996

A



B

